

REMARKS

Claims 1-8 and 11 have been amended.

Claims 14-20 have been added.

Claims 1-8 and 11 have been amended to clarify that the cachexia treated is “TNF α -mediated” cachexia. Support for these amendments is found in the specification, for example, at page 3, line 7-17; page 16, lines 15-26; and page 58, line 1 to page 59, line 14.

Claims 1 and 5 have been further amended to recite that the administered antibody competitively inhibits binding of “human TNF α ” to “anti-TNF α chimeric” monoclonal antibody cA2. Claim 1-3, 5-8 and 11 have been further amended to recite that the antibody administered is a “TNF α -inhibiting” amount of an “anti-TNF α ” chimeric antibody. Support for these amendments is found in the specification, for example, at page 10, lines 1-15 and page 16, lines 15-26.

Claim 4 has been further amended to recite “...administering to the human at least one anti-TNF α chimeric monoclonal antibody cA2, or a TNF α -binding fragment thereof.” Support for the amendment to Claim 4 is found in the specification, for example, at page 10, lines 1-15.

New Claim 14 is directed to the method of Claim 1, wherein said anti-TNF α antibody is administered to the human by means of parenteral administration. Support for New Claim 14 is found in the specification, for example, at page 59, line 23-29.

New Claim 15 is directed to the method of Claim 1, wherein said anti-TNF α chimeric antibody is administered to the human by means of intravenous administration, subcutaneous administration or intramuscular administration. New Claim 16 is directed to the method of Claim 1, wherein said anti-TNF α antibody is administered to the human via the lung. New Claim 17 is directed to the method of Claim 1, wherein said anti-TNF α antibody is administered to the human orally. Support for New Claims 15-17 is found in the specification, for example, at page 59, lines 23-29.

New Claim 18 is directed to the method of Claim 1, wherein said TNF α -inhibiting amount of the anti-TNF α antibody comprises a single or divided dose of about 0.1 - 50 mg/kg. New Claim 19 is directed to the method of Claim 18, wherein said single or divided dose is selected from the group consisting of: about a 0.1 - 1 mg/kg dose, about a 1.0 - 5 mg/kg dose, about a 5 - 10 mg/kg dose and about a 10 - 20 mg/kg dose. Support for New Claims 18 and 19 is

found in the specification, for example, at page 60, lines 7-24.

New Claim 20 is directed to the method of Claim 1, further comprising administering to the human an effective amount of a therapeutic agent selected from the group consisting of: radiotherapeutics, cytotoxic drugs, monoclonal antibodies, chimeric antibodies, antibody fragments, antibody regions, lymphokines, cytokines, hemopoietic growth factors and immunoglobulins. Support for New Claim 20 is found in the specification, for example, at page 62, line 17 to page 63, line 25.

No new matter has been added by the amendments. Therefore, entry of the amendments into the application is respectfully requested.

Examiner Interview

Applicants thank the Examiner for taking the time to discuss this case, and for providing helpful comments, during the Examiner interview at the United States Patent and Trademark Office held on June 16, 2005.

Correspondence Address

Please note that the undersigned Attorney has taken over responsibility for this application. A Notice of Change of Contact Attorney is being filed herewith.

Information Disclosure Statement

An Information Disclosure Statement (IDS) was filed on February 14, 2005. Entry of the IDS is respectfully requested.

Amendments to the Specification

The Examiner states that the application is to be reviewed and all spelling, trademarks, and like errors corrected, and that the first line of the specification should be amended to update the status of the priority documents.

Applicants have amended the specification to comply with the requirement to indicate trademarks, to correct typographical errors and to update the status of related applications. No new matter has been added by the amendments. Support for these amendments is found

throughout the specification. No new matter has been added by the amendments. Therefore, entry of the amendments into the application is respectfully requested.

Priority Documents

The Examiner states "...that not all of the priority documents, particularly USSN 07/670,827 were available to the Examiner at this time." Applicants are enclosing a copy of priority document USSN 07/670,827. If the Examiner needs copies of additional priority documents, Applicants are willing to provide copies of the priority documents to the Examiner.

Priority

The Examiner states that "[i]t appears that claims 1 and 3-5 have a priority date at least of USSN 07/853,606, filed 3/18/92." The Examiner further states "[h]owever, the filing date of instant claims 2 and 6-11 are deemed to be the filing date of priority application 08/192,093, filed 2/4/94." Further, the Examiner states that Applicant is invited to clarify the support for priority of the instant claims in the lineage of priority documents. Lastly, the Examiner states that it is unclear how Applicants' WO 92/16553 publication fits into Applicant's priority lineage.

A. Priority Date of Claims 1 and 3-5

Claims 1 and 3-5 are entitled to claim the benefit priority application USSN 07/670,827 (filed March 18, 1991). This priority application teaches that the "[h]igh affinity chimeric anti-TNF α mAbs of the present invention, which have potent TNF α neutralizing activity, including TNF α -neutralizing fragments thereof, are useful as therapeutic agents for TNF α -mediated human disease...." (Page 10, lines 22-25 of the USSN 07/670,827 specification). USSN 07/670,827 also discloses treatment of a number of TNF α -mediated diseases, including cancer, cachexia, neoplastic disease and malignant disease involving TNF α -secreting tumors. (See, for example, page 10, line 22 to page 11, line 4 and page 39, line 20 to page 40, line 9).

In addition, the specification of this priority application teaches treatment of TNF α -mediated diseases with the claimed antibodies. (See USSN 07/670,827 page 3, line 21 to page 5, line 23; page 10, line 22 to page 11, line 9; and page 39, line 20 to page 44, line 20). Although there is not a specific example in the 07/670,827 specification directed to treatment of TNF α -

mediated cachexia associated with cancer, the mechanism of treatment would be the same regardless of the TNF α -mediated disease.

Therefore, the priority application 07/670,827 (filed March 18, 1991) provides sufficient written description and enablement for Applicants' claimed methods of treating cachexia associated with cancer, and Applicants are entitled to claim the benefit of it. This priority application has been properly referenced on page 1 of the specification in compliance with 35 U.S.C. § 120.

B. Priority Date of Claims 2 and 6-11

1. Claims 2 and 6-10 are entitled to claim the benefit of priority application USSN 07/853,606 (filed March 18, 1992)

Claims 2 and 6 recite "...at least one epitope included in amino acids between 87-108 or both 59-80 and 87-108 of SEQ ID NO.:1 of hTNF, as determined by Geysen epitope mapping comprising use of TNF decapeptide pins which overlap at every second amino acid and synthesized on polyethylene pins." Priority application USSN 07/853,606 (filed March 18, 1992) provides sufficient written description and enablement for Claims 2 and 6 (see, for example, the specification at page 7, lines 29-35; page 8, lines 18-23; page 13, lines 8-22; page 20, lines 19-33; Figures 13 and 15; and Example XIII, particularly pages 68 and 69).

Claims 7-10 are also entitled to claim the benefit of priority application USSN 07/853,606. Claims 7 and 8 differ from Claim 1, in that they recite "...a non-human variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO.:3 and SEQ ID NO.:5...." Claims 9 and 10 recite "...a nucleic acid sequence selected from the group consisting of SEQ ID NO.:2 and SEQ ID NO.:4." Priority application USSN 07/853,606 provides sufficient written description and enablement for Claims 7-10 (see, for example, the specification at page 12, lines 20-23; Figures 17A-B; page 24, lines 5-17). Please note that the SEQ ID NO. identifiers were later amended to correct an inadvertent error in the specification to clarify that Figure 17B (renumbered Figure 16B after amendment), is a nucleic acid sequence (SEQ ID NO.: 4) and corresponding amino acid sequence (SEQ ID NO.: 5) of the heavy chain variable region of the cA2 monoclonal antibody (see, for example, page 2 of the Amendment

filed in US Serial No.: 08/324,799 on March 14, 1997, as discussed in detail below). The nucleic acid sequence (SEQ ID NO: 2) and corresponding amino acid sequence (SEQ ID NO:3) of the light chain are also disclosed.

Moreover, the Examiner has cited WO 92/16553 in a prior art rejection. The Examiner states “[g]iven the prior art teachings [of WO 92/16553] drawn to the same chimeric cA2 antibodies and/or the same cA2 starting materials, the specific antibody regions comprising SEQ ID NOS: 2, 3, 4 and/or 5 would be inherent properties of said recombinant cA2 antibodies.” (Page 5 of the Office Action dated February 10, 2005) As discussed below, to qualify as prior art, a reference must meet the requirement of enablement. (MPEP at § 2121.01) Therefore, if Applicant’s disclosure in the PCT application (WO 92/16553) is sufficient to qualify as prior art, then Applicants’ disclosure in the March 18, 1992 U.S. priority application (USSN 07/853,606), which was filed on the same date as the PCT application (March 18, 1992) and which is substantially identical, is sufficient to support the claims, and the claims, at the very least, are entitled to the benefit of priority of the filing date of March 18, 1992.

2. Claim 11 is entitled to claim the benefit of priority application USSN 07/670,827 (filed March 18, 1991)

Claim 11 recites “...wherein said anti-TNF α chimeric antibody has epitopic specificity identical to monoclonal antibody cA2.” Priority application USSN 07/670,827 provides sufficient written description and enablement for Claim 11 (see, for example, the specification at page 39, lines 6-10 and Example X, particularly page 68, lines 1-2 (“This finding is consistent with the expected identical epitope specificity of cA2 and murine A2.”)).

Further, the Examiner at page 5 of the Office Action dated February 10, 2005, states “[g]iven the teaching of antibodies that bind the epitope that are recognized by the cA2 anti-TNF antibody as well as the cA2 antibody itself, [WO 92/16553] teaches antibodies that bind identical epitopes of the cA2 antibody.” Moreover, as discussed below, to qualify as prior art, a reference must meet the requirement of enablement. (MPEP at § 2121.01) Therefore, if Applicant’s disclosure in the PCT application (WO 92/16553) is sufficient to qualify as prior art, then

Applicants' disclosure in the March 18, 1992 U.S. priority application (USSN 07/853,606), which has substantially the same specification as WO 92/16553, is sufficient to enable the claims, and the claims, at the very least, are entitled to the benefit of priority of the filing date of March 18, 1992.

C. WO 92/16553

The Examiner states that it is unclear how Applicants' WO 92/16553 publication fits into Applicant's priority lineage. The PCT application (WO 92/16553) was filed March 18, 1992 and published October 1, 1992 and it also claims the benefit of priority to the same U.S. priority application (U.S. Serial No. 07/670,827) as the subject application. The PCT application is substantially identical to priority application U.S. Serial No. 07/853,606, which was filed on the same date as the PCT application (March 18, 1992). However, as indicated on page 1 of the specification of the subject pending application, the PCT application (WO 92/16553) is not part of Applicants' priority lineage.

Rejection to Claims 1, 3-5 and 11 Under 35 U.S.C. § 112, first paragraph

Claims 1, 3-5 and 11 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. On page 4, paragraph 5 and 6 of the pending Office Action, the Examiner states that:

Given the disclosure and the claims encompassing the instant cA2 antibody set forth in U.S. Patent No. 5,919,452; the conditions for the deposit of biological materials under 35 USC 112, first paragraph, with respect to cA2 appear to have been satisfied.

However, Applicant is required to make the record clear exactly what is the scope of the instantly claimed cA2 and whether applicant has satisfied the deposit requirements under 35 USC 112, first paragraph, for the claimed cA2 antibody.

A. The Record Regarding Enablement Of The Claims Regarding The cA2 Antibody.

Applicants note that the following related patent applications have been examined and were determined to enable claims reciting the cA2 antibody and have issued as patents: US Serial No.: 08/192,102, now US Patent No.: 5,656,272; US Serial No. 08/192,861, now US Patent No. 5,919,452; US Serial No. 08/192,093, now US Patent No.: 6,284,471; and US Serial No.: 08/324,799, now US Patent No.: 5,698,195.

In these related applications, (see, for example, US Serial No.: 08/324,799, Amendment filed March 14, 1997) Applicants demonstrated that the claimed invention is enabled because the sequences of the heavy chain variable region and the light chain variable region of the cA2 monoclonal antibody are disclosed. A copy of the argument on pages 15-16 of the Amendment, and a copy of The Notice of Allowability, are enclosed herein as Exhibits A and B, respectively. For example, Applicants pointed out that Figure 16B is a nucleic acid sequence (SEQ ID NO:4) and corresponding amino acid sequence (SEQ ID NO:5) of a cloned cA2 heavy chain variable region. In addition, Applicants pointed out that Figure 16A is a nucleic acid sequence (SEQ ID NO:2) and corresponding amino acid sequence (SEQ ID NO:3) of a cloned cA2 light chain variable region. Thus, Applicants demonstrated that the claimed invention is enabled by the Specification and a deposit is not required.

Specifically, Applicants amended an inadvertent error in the Specification to clarify that Figure 17B (renumbered Figure 16B after amendment), is a nucleic acid sequence (SEQ ID NO.: 4) and corresponding amino acid sequence (SEQ ID NO.: 5) of the heavy chain variable region of the cA2 monoclonal antibody (see page 2 of the Amendment).

In addition, Applicants noted that:

Furthermore, in addition to disclosing the heavy and light chain variable sequences, Applicants have sufficiently disclosed are methods of production of chimeric anti-TNF antibodies which would be chemically and structurally similar to those claimed. Applicants' 176-page Specification provides ample instruction to one of skill in the art on how to produce and select antibodies for use in the claimed invention. See, for example, Examples I-X on page 82, line 6 through page 95, line 10.

"No deposit is necessary if the biological organisms can be obtained from readily available sources or derived from readily available starting materials through routine screening that does not require undue experimentation." In re Wands, 8

U.S.P.Q.2d 1400, 1403 (Fed. Cir. 1988). Since the Specification provides significant description of the properties (e.g., glycosylation, epitopic specificity and affinity) of the chimeric anti-TNF antibodies, the screening of antibodies which have the same or similar properties does not require undue experimentation. Please note that, to the best of knowledge of the undersigned, neither c134A nor the c168A cell line has been deposited in the ATCC collection.

Pages 15-16, US Serial No.: 08/324,799, Amendment filed March 14, 1997

In response, the Examiner withdrew the rejection, and, after an Examiner interview resulting in a claim amendment regarding fragments, allowed the claims. Such arguments and evidence apply equally to the subject application. Further, the arguments in response to similar enablement rejections in related patent applications USSN 08/192,093 and USSN 08/192,861, now US Patent No. 6,284,471 and US Patent No. 5,919,452, respectively, are essentially the same.

B. A Deposit Is Not Required Because The Disclosure Is Sufficient To Enable Production Of The Claimed Antibodies

In addition to the fact that related patent applications have been determined to be enabled and issued as patents, the cA2 antibodies can be obtained from publicly available material with only routine experimentation and a reliable screening test. Applicants direct the Examiner's attention to the Federal Circuit decision in *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988) (a copy of which is attached as Exhibit C for the Examiner's convenience). The claims at issue in *In re Wands* recited methods for an immunoassay using high affinity monoclonal antibodies that the Appellants found to have unexpectedly high sensitivity and specificity. The position of the PTO was that the data showed that the production of the antibodies is unpredictable and unreliable, so that it would require undue experimentation for one skilled in the art to make them. However, the court in *In re Wands* disagreed, noting that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening," as long as the experimentation was not undue. *Id.* at 1404. The court concluded that undue experimentation would not be required to practice the claimed invention.

The court first stated that "Wands' disclosure provides considerable direction and guidance on how to practice their invention and presents working examples." *Id.* at 1406. The

court further stated that "[t]here was a high level of skill in the art at the time when the application was filed, and all of the methods needed to practice the invention were well known."

Id. The court in *In re Wands* recognized that the nature of monoclonal antibody technology is such that it involves screening hybridomas to determine which ones secrete antibodies with desired characteristics, and that practitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody. *Id.* The court went on to state that "in the monoclonal antibody art it appears that an 'experiment' is not simply the screening of a single hybridoma, but is rather the entire attempt to make a monoclonal antibody against a particular antigen." *Id.* at 1407.

In considering the factors enumerated in *In re Wands*, Applicants' disclosure provides considerable direction and guidance on how to practice their invention, and presents numerous working examples. For example, the sequences of the variable regions of the antibodies are disclosed in Figures 16A-16B. In addition, Applicants disclose that the structural element of the constant region is human IgG1. Chimeric A2 (cA2) anti-TNF α antibody consists of the antigen binding variable region of the high-affinity neutralizing mouse antihuman TNF IgG1 antibody, designated A2, and the constant regions of a human IgG1, kappa immunoglobulin. The avidity and epitope specificity of the chimeric A2 is derived from the variable region of the murine A2. Chimeric A2 neutralizes the cytotoxic effect of both natural and recombinant human TNF. (See, for example, instant Detailed Description at page 34, line 10 to page 35, line 4). Further, the specification teaches methods of producing the claimed cA2 antibodies according to the present invention (See, for example, instant Detailed Description at page 32, lines 7 through 24; page 34, line 10 through page 35, line 4; and Examples III-IX). Examples I-IX teach the production, characterization and expression of the cA2 antibody. Examples X-XII teach assays for screening the cA2 antibody.

Additionally, it teaches methods of cloning a polynucleotide encoding an anti-TNF variable or constant region. (See, for example, instant Detailed Description at page 28, line 3 through page 31, line 2).

Furthermore, the instant specification teaches that preferred anti-TNF monoclonal antibodies include those which will competitively inhibit *in vivo* the binding to human TNF α of anti-TNF α murine monoclonal antibody A2, chimeric monoclonal antibody cA2, or an antibody

having substantially the same specific binding characteristics, as well as fragments and regions thereof. (See, for example, page 19, lines 17-20). It also teaches preferred methods for determining monoclonal antibody specificity and affinity (See, for example, instant Specification at page 19, line 25 through page 20, line 2, and Examples X and XI).

Thus, a person of skill in the art would not be subject to undue experimentation without a reasonable expectation of success in order to make and screen cA2 antibodies which would have these claimed elements. In addition, there was a high level of skill in the art at the time when the application was filed, and all of the methods needed to practice the invention were well known.

As discussed above, the instant Specification and figures, together with what was known and available in the art, provide ample teachings such that one of skill in the art would not be subject to undue experimentation in order to make or use the claimed antibodies. Thus, the skilled artisan is enabled to make and use the claimed invention commensurate in scope with the claims.

Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claims 1-11 Under 35 U.S.C. § 112, first paragraph

The Examiner has rejected Claims 1-11 under 35 U.S.C. § 112, first paragraph, "because the specification, while being enabling for the 'TNF α -specificity'; does not reasonably provide enablement for any 'TNF-specificity' having such specificities."

Applicants respectfully disagree. As noted above, to expedite prosecution, Applicants have amended Claims 1-8 and 11 to recite that the claimed antibodies are anti-TNF α antibodies. Applicants have exemplified that the cA2 antibody competitively inhibits and binds with high affinity a neutralizing epitope of human TNF- α . Therefore, particularly as amended, the claims are enabled.

However, it should also be noted that anti-TNF α antibodies are not the only antibodies supported by the specification. As indicated in the specification, the present invention provides anti-TNF compounds and compositions comprising anti-TNF antibodies (Abs) and/or anti-TNF peptides which inhibit and/or neutralize TNF biological activity *in vitro*, *in situ* and/or *in vivo*, as specific for association with neutralizing epitopes of human tumor necrosis factor-alpha (hTNF α) and/or human tumor necrosis factor β (hTNF β). (Page 16, lines 15-19.) Reconsideration and

withdrawal of the rejection are respectfully requested.

Rejection to Claims 1, 3-5 and 11 Under 35 U.S.C. § 112, second paragraph

The Examiner has rejected Claims 1, 3-5 and 11 as indefinite in the recitation of "cA2". Specifically, the Examiner states that "the use of 'cA2' antibody as the sole means of identifying the claimed antibody renders the claim indefinite because 'cA2' is merely a laboratory designation which does not clearly define the claimed product, since different laboratories may use the same laboratory designation [] to define completely distinct hybridomas / cell lines."

Applicants respectfully traverse this rejection.

Applicants note that the cA2 antibody is the same cA2 antibody claimed in related issued patents (US Patent No.: 5,656,272; US Patent No. 5,919,452; US patent No.: 6,284,471; and now US Patent No.: 5,698,195). For example, the claims of related U.S. Patent No. 6,284,471, which has the same priority date and has a substantially identical specification as the instant application, recite cA2. (A copy of the claim set of U.S. Patent No. 6,284,471 is attached hereto as "Exhibit I" for the Examiner's convenience). In related US Serial No. 08/192,861, now US Patent No. 5,919,452, this indefiniteness rejection was raised. In the Amendment filed on July 8, 1998, Applicants stated that:

As discussed in several previous responses in this case, the cA2 antibody has not been deposited with the ATCC, and therefore, it has no ATCC accession number. As stated in the Manual of Patent Examining Procedure, § 2172.01, "[a] fundamental principle contained in 35 U.S.C. § 112, Second Paragraph, is that Applicants are their own lexicographer." In addition, "a claim may not be rejected solely because of the type of language used to define the subject matter for which the patent protection is sought" § 2173.01. Applicants, in acting as their own lexicographers, created the designation cA2 for the specific chimeric anti-TNF α antibody disclosed in the application. The specification discloses activities of the antibody and the DNA (SEQ ID NOS: 2-5) and amino acid sequences of the heavy and light chain variable and constant regions of the cA2 monoclonal antibody in Figures 16A and 16B. The specification thereby defines the antibody and enables a person of ordinary skill in the art to produce cA2 and/or screen antibodies which have the same or similar properties without undue experimentation.

In response to the Amendment, the Examiner withdrew the rejection and allowed the claims. A copy of page 5, line 5 through page 6, line 29, from the Amendment filed July 8, 1998 for US Serial No. 08/192,861 and copy of the Notice of Allowability responsive to the Amendment are enclosed herewith as Exhibits D and E, respectively.

The claims and specification provide a great deal of description regarding cA2's structure and properties. As amended, the claims explicitly state that the antibody is a chimeric anti-TNF α monoclonal antibody. Further, the specification clearly discloses that the antibody is a chimeric anti-TNF α monoclonal antibody, and provides a detailed disclosure of the production, structure and function of cA2. (Specification at page 17, lines 2-8; page 19, lines 7-16; page 26, lines 21-28 and page 34, line 12 to page 35, line 4) For instance, Examples I-IX teach the production, characterization and expression of the cA2 antibody and Examples X-XII teach assays for screening the cA2 antibody for specificity and efficacy. Moreover, "cA2" is recognized by those skilled in the art as a unique identifier of Applicants' chimeric anti-TNF α monoclonal antibody. A number of scientific articles and press releases refer to Applicants' claimed monoclonal antibody as "cA2." (See, for example, Elliott, M. J. *et al.*, "Treatment of Rheumatoid Arthritis with Chimeric Monoclonal Antibodies to Tumor Necrosis Factor α ," *Arthritis Rheum*, 36:1681-1690 (1993) (Exhibit F); Walker, R.E., "Inhibition of Immunoreactive Tumor Necrosis Factor-alpha by a Chimeric Antibody in Patients Infected with Human Immunodeficiency Virus Type 1," *J. Infect. Dis.*, 174(1):63-8 (1996), abstract from AIDSLINEMED/96261994 (Exhibit G); and "New Monoclonal Antibody Effective Treatment For Crohn's Disease Therapy," Doctor's Guide (May 13, 1997), <http://www.docguide.com/dg.nsf/PrintPrint/815D53A771190A4285256496004B0796> (Exhibit H)). These references are representative of the general knowledge of one skilled in the art and demonstrate that the identifier "cA2" clearly defines the claimed product. Thus, the cA2 antibody is well known in the art.

Thus, the term cA2 is not indefinite.

Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection to Claims 2 and 6-11 Under 35 U.S.C. § 102(b)

The Examiner has rejected Claims 2 and 6-11 under 35 U.S.C. § 102(b) as being

anticipated by Le *et al.* (WO 92/16553). The Examiner states that:

Le et al. teach treating cachexia associated with cancer in humans...by administering the recombinant cA2 antibody of the instant invention.... In addition, Le et al. teach the determination of amino acid sequence of cA2-specific epitopes via Geysen epitope mapping.... Given the teaching of antibodies that bind the epitope that are recognized by the cA2 anti-TNF antibody as well as the cA2 antibody itself, the prior art teaches antibodies that bind the identical epitope of the cA2 antibody. Given the prior art teachings drawn to the same chimeric cA2 antibodies and/or the same cA2 starting materials, the specific antibody regions comprising SEQ ID NOS: 2, 3, 4 and 5 would be inherent properties if said recombinant cA2 antibodies.

1. Claims 2 and 6-10 are entitled to claim the benefit of priority application USSN 07/853,606 (filed March 18, 1992)

As discussed in the above section regarding priority, Claims 2 and 6-10 are entitled to claim the benefit of priority application USSN 07/853,606 (filed March 18, 1992). Le *et al.* (WO 92/16553) is not prior art under 35 U.S.C. § 102 (b) because it was not published more than one year before Applicants' priority date. The PCT application (WO 92/16553) was published on October 1, 1992 and it is substantially identical to U.S. Serial No. 07/853,606, which was filed on the same date as the PCT application (March 18, 1992).

To qualify as prior art, a reference must meet the requirement of enablement. As stated in the MPEP at § 2121.01:

“In determining that quantum of prior art disclosure which is necessary to declare an applicant's invention ‘not novel’ or ‘anticipated’ within section 102, the stated test is whether a reference contains an ‘enabling disclosure’”

(Quoting *In re Hoeksema*, 399 F.2d 269, 158 USPQ 596 (CCPA 1968)).

The disclosure in an assertedly anticipating reference must provide an enabling disclosure of the desired subject matter; mere naming or description of the subject matter is insufficient, if it cannot be produced without undue experimentation.

(Citing *Elan Pharm. Inc. v. Mayo Foundation for Medical and Education Research*, 346 F.3d

1051, 1054, 68 USPQ2d 1373, 1376 (Fed. Cir. 2003).

Therefore, if *Le et al.*'s disclosure, in the PCT application (WO 92/16553), is sufficient to qualify as prior art, then Applicants' March 18, 1992 priority application (USSN 07/853,606) is sufficient to enable the claims. In addition, as indicated above, Applicants' March 18, 1992 priority application provides sufficient written description. Thus, the claims, at the very least, are entitled to the benefit of priority to the filing date of March 18, 1992. Hence, Applicants' PCT application *Le et al.* (WO 92/16553) is not prior art.

Thus, reconsideration and withdrawal of the rejection are respectfully requested.

2. Claim 11 is entitled to claim the benefit of priority application USSN 07/670,827 (filed March 18, 1991)

Le et al. (WO 92/16553) is not prior art under 35 U.S.C. § 102 (b) because it was not published more than one year before Applicants' priority date. Claim 11, as discussed above, is entitled to priority to U.S. Serial No. 07/670,827 (filed March 18, 1991). Furthermore, the PCT application was published on October 1, 1992 and is substantially identical to the corresponding U.S. priority application (U.S. Serial No. 07/853,606) of the subject application, which was filed on the same date as the PCT application (March 18, 1992).

As indicated above, Applicants' March 18, 1992 priority application provides sufficient written description. The Examiner at page 5 of the Office Action dated February 10, 2005, states "[g]iven the teaching of antibodies that bind the epitope that are recognized by the cA2 anti-TNF antibody as well as the cA2 antibody itself, [WO 92/16553] teaches antibodies that bind identical epitopes of the cA2 antibody." Moreover as discussed above, if Applicant's disclosure in the PCT application (WO 92/16553) is sufficient to qualify as prior art, then Applicants' disclosure in the March 18, 1992 U.S. priority application (USSN 07/853,606) is sufficient to enable the claims. Thus, the claims at the very least, are entitled to the benefit of priority to the filing date of March 18, 1992.

Hence, *Le et al.* (WO 92/16553) is not prior art.

Thus, reconsideration and withdrawal of the rejection are respectfully requested.

Non-statutory Double Patenting Rejection

The Examiner states that "Applicant is invited to clarify which applications should be subject to rejections under the judicially created doctrine of obviousness-type double patenting."

Applicants do not believe that any of their co-pending applications are subject to any obviousness-type double patenting rejection.

Provisional Rejection of Claims 1-11 Under Obviousness-type Double Patenting

The Examiner has provisionally rejected Claims 1-11 "under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-16 of copending USSN 10/957,134 and Claims 1-70 of copending USSN 10/957,549."

If necessary, Applicants will file a terminal disclaimer upon resolution of the remaining rejections.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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COPY



MONOCLONAL AND CHIMERIC ANTIBODIES SPECIFIC FOR HUMAN TUMOR NECROSIS FACTOR

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

5 The invention in the field of immunology and medicine
relates to monoclonal and chimeric antibodies specific for human
tumor necrosis factor-alpha (hTNF α), their fragments and
derivatives, pharmaceutical compositions containing them, methods
of producing them, uses for the antibodies in diagnosis and
10 therapy of human disease. The invention further relates to
nucleotide sequences encoding the chimeric antibodies, and
vectors and hosts containing the sequences.

DESCRIPTION OF THE BACKGROUND ART

General Properties of TNF and Its Role in Disease

15 The cytokine known as tumor necrosis factor-a (TNF;
also termed cachectin) is a protein secreted primarily by
monocytes and macrophages in response to endotoxin or other
stimuli as a soluble homotrimer of 17 kD protein subunits (Smith,
R.A. et al., J. Biol. Chem. 262:6951-6954 (1987)). A membrane-
20 bound 26 kD precursor form of TNF has also been described
(Kriegler, M. et al., Cell 53:45-53 (1988)). For reviews of TNF,
see Beutler, B. et al., Nature 320:584 (1986), Old, L.J., Science
230:630 (1986), and Le, J. et al., Lab. Invest. 56:234 (1987).
TNF was originally discovered in the serum of animals injected

sequentially with a bacterial vaccine (bacillus Calmette-Guerin, BCG) and endotoxin (Carswell, E.A. et al., Proc. Natl. Acad. Sci. USA 72:3666 (1975)).

5 The expression of the gene encoding TNF is not limited to cells of the monocyte/macrophage family. Several human non-monocytic tumor cell lines were shown to produce TNF (Rubin, B.Y. et al., J. Exp. Med. 164:1350 (1986); Spriggs, D. et al., Proc. Natl. Acad. Sci. USA 84:6563 (1987)). TNF is also produced by CD4⁺ and CD8⁺ peripheral blood T lymphocytes, and by various
10 cultured T and B cell lines (Cuturi, M.C., et al., J. Exp. Med. 165:1581 (1987); Sung, S.-S.J. et al., J. Exp. Med. 168:1539 (1988)).

Accumulating evidence indicates that TNF is a regulatory cytokine with pleiotropic biological activities.
15 These activities include: inhibition of lipoprotein lipase synthesis ("cachectin" activity) (Beutler, B. et al., Nature 316:552 (1985)), activation of polymorphonuclear leukocytes (Klebanoff, S.J. et al., J. Immunol. 136:4220 (1986); Perussia, B., et al., J. Immunol. 138:765 (1987)), inhibition of cell
20 growth or stimulation of cell growth (Vilcek, J. et al., J. Exp. Med. 163:632 (1986); Sugarman, B.J. et al., Science 230:943 (1985); Lachman, L.B. et al., J. Immunol. 138:2913 (1987)), cytotoxic action on certain transformed cell types (Lachman, L.B. et al., supra; Darzynkiewicz, Z. et al., Canc. Res. 44:83
25 (1984)), antiviral activity (Kohase, M. et al., Cell 45:659 (1986); Wong, G.H.W. et al., Nature 323:819 (1986)), stimulation

of bone resorption (Bertolini, D.R. et al., Nature 319:516 (1986); Saklatvala, J., Nature 322:547 (1986)), stimulation of collagenase and prostaglandin E2 production (Dayer, J.-M. et al., J. Exp. Med. 162:2163 (1985)); and immunoregulatory actions, including activation of T cells (Yokota, S. et al., J. Immunol. 140:531 (1988)), B cells (Kehrl, J.H. et al., J. Exp. Med. 166:786 (1987)), monocytes (Philip, R. et al., Nature 323:86 (1986)), thymocytes (Ranges, G.E. et al., J. Exp. Med. 167:1472 (1988)), and stimulation of the cell-surface expression of major histocompatibility complex (MHC) class I and class II molecules (Collins, T. et al., Proc. Natl. Acad. Sci. USA 83:446 (1986); Pujol-Borrell, R. et al., Nature 326:304 (1987)).

TNF is noted for its pro-inflammatory actions which result in tissue injury, such as induction of procoagulant activity on vascular endothelial cells (Pober, J.S. et al., J. Immunol. 136:1680 (1986)), increased adherence of neutrophils and lymphocytes (Pober, J.S. et al., J. Immunol. 138:3319 (1987)), and stimulation of the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi, G. et al., J. Exp. Med. 166:1390 (1987)).

Recent evidence implicates TNF in the pathogenesis of many infections (Cerami, A. et al., Immunol. Today 9:28 (1988)), immune disorders, neoplastic disease, e.g., in cachexia accompanying some malignancies (Oliff, A. et al., Cell 50:555 (1987)), and in autoimmune diseases and graft-versus host disease (Piguet, P.-F. et al., J. Exp. Med. 166:1280 (1987)). The

association of TNF with cancer and infectious diseases is often related to the host's catabolic state. A major problem in cancer patients is weight loss, usually associated with anorexia.

The extensive wasting which results is known as "cachexia"

5 (Kern, K.A. et al. (J. Parent, Enter. Nutr. 12:286-298 (1988))).

Cachexia includes progressive weight loss, anorexia, and persistent erosion of body mass in response to a malignant growth. The fundamental physiological derangement may be related to a decline in food intake relative to energy expenditure. The
10 cachectic state is thus associated with significant morbidity and is responsible for the majority of cancer mortality. A number of studies have suggested that TNF is an important mediator of the cachexia in cancer, infectious disease, and in other catabolic states.

15 TNF is thought to play a central role in the pathophysiological consequences of Gram-negative sepsis and endotoxic shock (Michie, H.R. et al., Br. J. Surg. 76:670-671 (1989); Debets, J.M.H. et al., Second Vienna Shock Forum, p.463-466 (1989); Simpson, S.Q. et al., Crit. Care Clin. 5:27-
20 47 (1989)), including fever, malaise, anorexia, and cachexia.

Endotoxin is a potent monocyte/macrophage activator which stimulates production and secretion of TNF (Kornbluth, S.K. et al., J. Immunol. 137:2585-2591 (1986)) and other cytokines.

Because TNF could mimic many biological effects of endotoxin, it
25 was concluded to be a central mediator responsible for the clinical manifestations of endotoxin-related illness. TNF and

other monocyte-derived cytokines mediate the metabolic and neurohormonal responses to endotoxin (Michie, H.R. et al., N. Eng. J. Med. 318:1481-1486 (1988)). Endotoxin administration to human volunteers produces acute illness with flu-like symptoms including fever, tachycardia, increased metabolic rate and stress hormone release (Revhaug, A. et al., Arch. Surg. 123:162-170 (1988)). Elevated levels of circulating TNF have also been found in patients suffering from Gram-negative sepsis (Waage, A. et al., Lancet 1:355-357 (1987); Hammerle, A.F. et al., Second Vienna Shock Forum p. 715-718 (1989); Debets, J.M.H. et al., Crit. Care Med. 17:489-497 (1989); Calandra, T. et al., J. Infec. Dis. 161:982-987 (1990)). Treatment of cancer patients with TNF (because of its tumoricidal action) revealed that doses greater than 545 µg/m²/24hr caused alterations similar to those induced by injection of endotoxin (4 ng/kg) into healthy humans (Michie, H.R. et al., Surgery 104:280-286 (1988)), supporting TNF's role as the principal host mediator of septic and endotoxemic responses. Chronic intravenous TNF infusion into humans or rats was associated with anorexia, fluid retention, acute phase responses, and negative nitrogen balance (i.e., classic catabolic effects), leading to the conclusion that TNF may be responsible for many of the changes noted during critical illness (Michie, H.R. et al., Ann. Surg. 209:19-24 (1989)).

Antibodies to TNF and Their Use in Diagnosis and Therapy

Passive immunotherapy directed at neutralizing TNF may have a beneficial effect in Gram-negative sepsis and endotoxemia, based on the increased TNF production and elevated TNF levels in these disease states, as discussed above.

5 Antibodies to a "modulator" material, which was characterized as cachectin (later found to be identical to TNF) were disclosed by Cerami et al. (EPO Patent Publication 0212489, March 4, 1987). Such antibodies were said to be useful in diagnostic immunoassays and in therapy of shock in bacterial
10 infections. Rubin et al. (EPO Patent Publication 0218868, April 22, 1987) disclosed monoclonal antibodies to human TNF, the hybridomas secreting such antibodies, methods of producing such antibodies, and the use of such antibodies in immunoassay of TNF. Yone et al. (EPO Patent Publication 0288088, October 26, 1988)
15 disclosed anti-TNF antibodies, including mAbs, and their utility in immunoassay diagnosis of diseases, in particular Kawasaki's disease and bacterial infection. The body fluids of patients with Kawasaki's disease (infantile acute febrile mucocutaneous lymph node syndrome; Kawasaki, T., Allergy 16:178 (1967);
20 Kawasaki, T., Shonica (Pediatrics) 26:935 (1985)) were said to contain elevated TNF levels which were related to progress of the disease (Yone et al., supra).

Other investigators have described mAbs specific for recombinant human TNF which had neutralizing activity in vitro
25 (Liang, C-M. et al., (Biochem. Biophys. Res. Comm. 137:847-854 (1986); Meager, A. et al., Hybridoma 6:305-311 (1987); Fendly et

al., Hybridoma 6:359-369 (1987); Bringman, T.S. et al.,
Hybridoma 6:489-507 (1987); Hirai, M. et al., J. Immunol. Meth.
96:57-62 (1987); Moller, A. et al. (Cytokine 2:162-169 (1990)).

Some of these mAbs were used to map epitopes of human TNF and
5 develop enzyme immunoassays (Fendly et al., supra; Hirai et al.,
supra; Moller et al., supra) and to assist in the purification of
recombinant TNF (Bringman et al., supra).

The most direct support for the use of anti-TNF
immunotherapy comes from in vivo protection studies in animals.

10 Neutralizing antisera or mAbs to TNF have been shown to abrogate
adverse physiological changes and prevent death after lethal
challenge in experimental endotoxemia and bacteremia animal model
systems. This effect has been demonstrated in rodent lethality
assays and in primate disease model systems (Mathison, J.C. et
15 al., J. Clin. Invest. 81:1925-1937 (1988); Beutler, B. et al.,
Science 229:869-871 (1985); Tracey, K.J. et al., Nature 330:662-
664 (1987); Shimamoto, Y. et al., Immunol. Lett. 17:311-318
(1988); Silva, A.T. et al., J. Infect. Dis. 162:421-427 (1990);
Opal, S.M. et al., J. Infect. Dis. 161:1148-1152 (1990); Hinshaw,
20 L.B. et al., Circ. Shock 30:279-292 (1990)). For example,
F(ab')₂ fragments of neutralizing anti-TNF mAbs were able to
prevent septic shock produced by live E. coli in baboons (Tracey,
K.J. et al., supra).

To date, experience with anti-TNF mAb therapy in humans
25 has been limited. In a phase I study, fourteen patients with
severe septic shock administered a neutralizing mouse anti-TNF

mAb in a single dose from 0.4-10 mg/kg (Exley, A.R. et al.,
Lancet 335:1275-1277 (1990)), and the treatment was concluded to
be safe and without acute side-effects. Seven of the fourteen
patients developed a human anti-murine antibody response to the
5 treatment. Administration of murine TNF mAb to patients
suffering from severe graft vs. host disease has also been
reported (Herve, P. et al., Lymphoma Res. 9:591 (1990)). While
the results of this small study were too preliminary for
efficacy interpretation, no acute side-effects of the treatment
10 were noted.

Monoclonal and Chimeric Antibodies

Monoclonal antibody technology has spawned a revolution
in biology equal in impact to that of recombinant DNA technology.
MAbs produced by hybridoma cells are already widely used in
15 diagnostics and in basic biomedical research. Their success in
the treatment of human diseases, including microbial infections,
autoimmune disease, and cancer, has yet to be established.
Despite their exquisite specificity, mouse mAbs, by their very
nature, have limitations in their applicability to human
20 medicine. Most obviously, due to their murine origin, they are
foreign proteins in humans, induce anti-murine immune responses
and tend to be cleared more rapidly from the circulation. Since
treatment of many diseases would require multiple administrations
of a mAb, the risk of immune reactions, including anaphylaxis,
25 may severely hamper the widespread applicability of murine mAbs

in most of the human diseases discussed above. Finally, mouse antibodies may not interact as effectively as human antibodies with human effector cells (e.g., macrophages) or with human effector molecules (e.g., complement components).

5 The development of human mAbs that could circumvent the above problems has encountered a number of obstacles. Because human spleen cell donors are rare, human mAb-producing cell lines are typically obtained from human peripheral B cells immortalized by infection with Epstein Barr Virus (EBV). Such
10 cells may not be useful for scale-up and production of human pharmaceuticals. Furthermore, the presence of human viral genetic information in such EBV-immortalized human antibody-producing cell lines may be a barrier to their safe use. In addition, since human TNF has evolved in the face of the human
15 immune response, key antigenic epitopes may not be recognized by the human immune system. Such antigens would not be expected to elicit useful immune responses in man. In contrast, those human antigens that are immunogenic in mice can be used for the production of mouse mAbs which may have the appropriate antigen
20 specificity to be of therapeutic utility in humans.

A chimeric antibody may comprise an antigen binding region of non-human origin, such as that derived from a murine mAb, joined to a human constant region. Use of such chimeric antibodies has been suggested as a means for avoiding the problem
25 of immunogenicity of murine antibodies in humans. Since the constant region is largely responsible for immunogenicity of an

antibody molecule, chimeric antibodies with human constant regions are less likely to evoke an immune response in a human. Chimeric antibodies and methods for their production have been described in the scientific and patent literature (Cabilly et al.,
5 Proc. Natl. Acad. Sci. USA 81:3273-3277 (1984); Morrison et al.,
Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Boulianne
et al., Nature 312:643-646 (1984); Neuberger et al., Nature
314:268-270 (1985); Taniguchi et al., European Patent Publication
171496 (February 19, 1985); Robinson et al., International Patent
10 Publication PCT/US86/02269 (May 7, 1987); Liu, et al., Proc.
Natl. Acad. Sci. USA 84:3439-3443 (1987); Better et al., Science
240:1041-1043 (1988); LoBuglio, A. et al., Proc. Natl. Acad. Sci.
USA 86:4220 (1989); LoBuglio et al., International Patent
Publication PCT/GB85/00392).

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SUMMARY OF THE INVENTION

Chimeric antibody technology, such as that used in the present invention for producing chimeric antibodies specific for human tumor necrosis factor-alpha (hTNF α), bridges both the
20 hybridoma and genetic engineering technologies to provide useful products for the treatment and diagnosis of human diseases.

High affinity chimeric anti-TNF α mAbs of the present invention, which have potent TNF α neutralizing activity, including TNF α -neutralizing fragments thereof, are useful as
25 therapeutic agents for TNF α -mediated human disease, including

cachexia, acute and chronic infectious and parasitic processes, such as bacterial, viral and fungal infections, acute and chronic inflammatory and immune processes, including autoimmune disease, alcohol-induced hepatitis, neoplastic disease and the like.

5 Preferred for therapeutic use are high affinity chimeric anti-TNF α antibodies according to the present invention, which neutralize human TNF- α with an inhibitory dose-50 (ID50) of at least about 1 μ g/ml, more preferably at least about 100 ng/ml, most preferably at least about 15 ng/ml.

10 High affinity chimeric anti-TNF α mAbs of the present invention, or TNF α -binding fragments thereof, are also particularly useful in diagnostic methods for detecting human TNF α in patients suspected of suffering from conditions associated with TNF α production, including methods wherein high
15 affinity murine and/or chimeric anti-TNF α antibodies of the present invention are contacted with biological materials from a patient and an antigen-antibody reaction detected. Also included are kits for detecting TNF α in biological fluids comprising high affinity murine and/or chimeric anti-TNF α antibodies or fragments
20 of the present invention, preferably in detectably labeled form.

The chimeric antibodies of the present invention embody a combination of the advantageous characteristics of mAbs. Like mouse mAbs, they can recognize and bind to human TNF; however, unlike mouse mAbs, the "human-specific" properties of
25 the chimeric antibodies lower the likelihood of an immune response to the antibodies, and result in prolonged survival in

the circulation through reduced clearance. Moreover, using the methods disclosed in the present invention, the constant region of any desired human immunoglobulin isotype can be combined with the desired antigen combining site.

5 In one embodiment, the present invention is directed to a high affinity mouse mAb, designated A2, which is specific for human TNF α . The antibody may be in detectably labeled form.

The invention provides chimeric immunoglobulin chains, either heavy (H) or light (L), with specificity toward one or
10 more epitopes of TNF. A chimeric immunoglobulin chain contains a constant (C) region substantially similar to that present in a natural human immunoglobulin, and a variable (V) region, preferably non-human, having high affinity and the desired specificity for a TNF epitope. The invention also provides
15 immunoglobulin molecules having chimeric H and L chains associated so that the overall molecule exhibits the desired antigen recognition and binding properties.

Specifically, the present invention is also directed to a chimeric antibody comprising two light chains and two heavy
20 chains, each of the chains comprising at least part of a human constant region and at least part of a variable (V) region of non-human origin having specificity to human TNF α , said antibody binding with high affinity to a neutralizing epitope of human TNF α . The invention is also includes a fragment or a derivative
25 such an antibody. Preferably, the V region is of non-human origin, most preferably of murine origin. In a preferred

embodiment, the V region is derived from the A2 mAb. In a most preferred embodiment, the chimeric antibody is the antibody designated chimeric A2 (cA2), or a chimeric human-mouse anti-TNF mAb that competitively inhibits the binding of cA2 to TNF α .

5 Preferably, the chimeric antibody has an affinity, measured as an association constant (K_a), of at least about 1×10^8 liter/mole, more preferably, at least about 1×10^9 liter/mole.

10 Preferably, the chimeric antibody neutralizes human TNF α with an ID₅₀ of at least about 1 μ g/ml, more preferably at least about 100 ng/ml, most preferably at least about 15 ng/ml.

In one embodiment the chimeric antibody is in detectably labeled form.

15 The present invention is further directed to a polynucleotide molecule comprising:

(a) a first nucleotide sequence encoding at least a part of the variable region of an immunoglobulin chain, either a heavy chain or a light chain, having specificity to human TNF α ; and

20 (b) a second nucleotide sequence encoding at least a part of the constant region of a human immunoglobulin chain, either a heavy chain or a light chain,

25 both the sequences in operable linkage with each other, wherein the polynucleotide molecule encodes a protein chain capable of yielding an antibody with high affinity binding to human TNF α .

The first and second nucleotide sequences may be either genomic DNA or cDNA sequences.

Preferably, the variable region of the polynucleotide molecule is of murine origin. In one embodiment it is derived from a murine mAb or murine hybridoma. In a most preferred embodiment, it is derived from the murine A2 hybridoma that produces the mAb A2 or from a murine hybridoma that produces a murine mAb that competitively inhibits the binding of mAb A2 to human TNF α .

In a preferred embodiment, the polynucleotide molecule is a recombinant DNA molecule. This molecule may be in double stranded DNA form. Included in the present invention is the molecule as an expression vehicle, such as a plasmid.

The present invention is also directed to a host transformed or transfected with the recombinant DNA polynucleotide molecule, preferably a eukaryotic host, in particular a mammalian cell such as a non-secreting human myeloma cell or other mammalian cell of B lymphocyte lineage.

The present invention includes a process for preparing a chimeric antibody comprising: (a) culturing a host capable of expressing the chimeric heavy and light chain wherein the heavy and light chain each has at least part of a human constant region and at least part of a non-human variable region having specificity to human TNF α ; (b) expressing the chimeric antibody; and (c) recovering the chimeric antibody from the culture.

In a more preferred process, a mammalian cell capable of

expressing intact immunoglobulins, such as a cell of B lymphocyte lineage, more preferably a non-secreting myeloma cell, is co-transfected with DNA encoding a chimeric heavy and a chimeric light chain. The transfected mammalian cell may then be grown
5 under conditions whereby chimeric heavy and light chains, as above, are co-expressed, assembled, glycosylated, and secreted, so that intact chimeric anti-TNF α immunoglobulins of this invention may be recovered.

The present invention also provides a method for
10 treating a subject having a disease or condition associated with increased production or secretion of TNF α , or mediated by TNF α , comprising administering to the subject a therapeutic amount of a monoclonal antibody or chimeric antibody described above.

The present invention is further directed to a method
15 of removing TNF, a TNF fragment, or an immune complex containing TNF or its fragment, from a fluid, comprising: (a) delivering the fluid to a device containing an immobilized monoclonal or chimeric antibody, as above; and (b) allowing binding of the TNF, antigenic fragment or immune complex to the immobilized antibody,
20 thereby removing the TNF, antigenic fragment or immune complex from the fluid. This method may be used in a method to treat a subject suspected of having a disease or condition associated with elevated levels of a substance reactive with anti-TNF antibodies, most particularly, TNF, in a body fluid, comprising:
25 (a) removing TNF from the body fluid as described above; and
(b) returning the body fluid to the subject.

The high affinity monoclonal and chimeric antibodies of the present invention are useful in an immunoassay method to detect human TNF in a biological sample, which comprises: (a) contacting the sample with a monoclonal or chimeric antibody as described above; and (b) detecting the binding of the antibody to the TNF.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing dose dependent binding of mouse mAb A2 to human TNF α .

Figure 2 is a graph showing lack of recognition of heat-inactivated human TNF α by mAb A2.

Figure 3 is a graph showing neutralization of in vitro TNF cytotoxicity by murine A2. Control: murine IgG1 anti-lipid A mAb (8A1) with natural human TNF. Average absorbance values for controls were as follows: no TNF added = 1.08; natural TNF, no antibody = 0.290; and recombinant TNF, no antibody = 0.500.

Figure 4 is a graph showing that mAb A2 and chimeric A2 do not neutralize human lymphotoxin (TNF β).

Figure 5 is a graph showing that mAbs murine A2 and chimeric CA2 do not neutralize murine TNF α .

Figures 6 and Figure 7 are graphs showing that mAb A2 neutralizes TNF produced by chimpanzee monocytes and rhTNF α .

Figure 8 provides schematic diagrams of the plasmids used for expression of the chimeric H (pA2HGlapgpt) and L (pA2HuKapgpt) chains of the chimeric A2 antibody.

Figure 9 is a graph showing results of a cross-blocking epitope ELISA with murine A2 (mA2) and chimeric (cA2) antibody competitors.

Figure 10 is a graph of a Scatchard analysis of ^{125}I -labelled mAb A2 (mA2) and chimeric A2 (cA2) binding to recombinant human TNF α immobilized on a microtiter plate. Each Ka value was calculated from the average of two independent determinations.

Figure 11 is a graph showing neutralization of TNF cytotoxicity by chimeric A2. The control is a chimeric mouse/human IgG1 anti-platelet mAb (7E3) reacting with natural human TNF. Average absorbance values for controls were: no TNF added = 1.08; natural TNF, no Ab = 0.290; and recombinant TNF, no Ab = 0.500.

Figure 12 is a graph showing in vitro neutralization of TNF-induced ELAM-1 expression by chimeric A2. The control is a chimeric mouse/human IgG1 anti-CD4 antibody.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides antibodies, including monoclonal and chimeric antibodies that are specific for, and capable of binding with high affinity to, human TNF α and which

can be used for diagnostic and therapeutic purposes in subjects having diseases or conditions associated with the presence of a substance reactive with anti-TNF antibody, in particular hTNF α , in amounts exceeding those present in a normal healthy subject.

5 The chimeric antibodies preferably contain mouse V regions which recognize an epitope of TNF, most preferably an epitope which is a "neutralizing epitope."

Preferred are high affinity human-murine chimeric anti-TNF α antibodies that have potent neutralizing activity against human TNF α . Since circulating concentrations of TNF tend to be extremely low, in the range of about 10 pg/ml in non-septic individuals, and reaching about 50 pg/ml in septic patients and above 100 pg/ml in the sepsis syndrome (Hammerle, A.F. et al., 1989, supra) or may be only be detectable at sites of TNF-mediated pathology, it is preferred to use high affinity and/or potent TNF-neutralizing antibodies for both TNF immunoassays and therapy of TNF-mediated disease. Such antibodies will preferably have an affinity for hTNF α , expressed as K_a , of at least 10^8 M^{-1} , more preferably, at least 10^9 M^{-1} .

20 Preferred for human therapeutic use are high affinity chimeric antibodies with potent TNF α -neutralizing activity, according to the present invention, that block TNF-induced IL-6 secretion. Also preferred for human therapeutic uses are such high affinity chimeric anti-TNF α antibodies that block TNF-induced procoagulant activity, including blocking of TNF-induced

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expression of cell adhesion molecules such as ELAM-1 and ICAM-1 and blocking of TNF mitogenic activity.

Preferred anti-TNF mAbs are those which will competitively inhibit the binding to human TNF α of anti-TNF α murine mAb A2, chimeric mAb cA2, or an antibody having substantially the same specific binding characteristics. The preferred antibodies of the present invention are A2 and cA2. Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Muller, R., Meth. Enzymol. 92:589-601 (1983), which is hereby incorporated by reference. Murine mAb A2 is produced by a cell line designated c134A. Chimeric antibody cA2 is produced by a cell line designated c168A.

Cell line c134A is deposited as a research cell bank in the Centocor Cell Biology Services Depository, and cell line c168A(RCB) is deposited as a research cell bank in the Centocor Corporate Cell Culture Research and Development Depository, both at Centocor, 200 Great Valley Parkway, Malvern, Pennsylvania, 19355. The c168A cell line is also deposited at Centocor BV, Leiden, The Netherlands.

Furthermore, c168A was deposited as of the filing date of the present application at the American Type Culture Collection, Rockville, Maryland, as a "Culture Safe Deposit."

The term "epitope" is meant to refer to that portion of any molecule capable of being recognized by and bound by an antibody. Epitopes usually consist of chemically active surface

groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. By "neutralizing epitope" is intended an epitope, which, when bound by an antibody, results in loss of biological activity of the molecule or organism containing the epitope.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The term "antibody" is meant to include both intact molecules as well as fragments and derivative thereof. Fragments include, for example, Fab, Fab', F(ab')₂, and Fv, which are capable of binding antigen. These fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)). These fragments are produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments).

In a preferred embodiment, the antibody is a monoclonal antibody designated A2. In another preferred embodiment, the antibody is a chimeric antibody which recognizes an epitope recognized by A2. In a most preferred embodiment, the antibody is a chimeric antibody designated as chimeric A2 (cA2).

The chimeric antibodies of the invention comprise individual chimeric heavy (H) and light (L) immunoglobulin chains. The chimeric H chain comprises an antigen binding region derived from the H chain of a non-human antibody specific for TNF, which is linked to at least a portion of a human H chain, C region (C_H).

A chimeric L chain according to the present invention, comprises an antigen binding region derived from the L chain of a non-human antibody specific for TNF, linked to at least a portion of a human L chain C region (C_L).

As used herein, the term "antigen binding region" refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues.

As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is tetramer (H_2L_2) formed by two HL

dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a C_H region that aggregates (e.g., from an IgM H chain, or μ chain).

5 The invention also provides for "derivatives" of the mouse mAbs or the chimeric antibodies, which term includes those proteins encoded by truncated or modified genes to yield molecular species functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to,
10 addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins. The fragments and derivatives can be produced from any of the hosts of this invention.

 Antibodies, fragments or derivatives having chimeric H chains and L chains of the same or different V region binding
15 specificity, can be prepared by appropriate association of the individual polypeptide chains, as taught, for example by Sears et al., Proc. Natl. Acad. Sci. USA 72:353-357 (1975). With this approach, hosts expressing chimeric H chains (or their derivatives) are separately cultured from hosts expressing
20 chimeric L chains (or their derivatives), and the immunoglobulin chains are separately recovered and then associated. Alternatively, the hosts can be co-cultured and the chains allowed to associate spontaneously in the culture medium, followed by recovery of the assembled immunoglobulin, fragment or
25 derivative.

The antigen binding region of the chimeric antibody of the present invention is derived preferably from a non-human antibody specific for human TNF. Preferred sources for the DNA encoding such a non-human antibody include cell lines which produce antibody, preferably hybrid cell lines commonly known as hybridomas. A preferred hybridoma is the A2 hybridoma cell line. The hybrid cells are formed by the fusion of a non-human anti-hTNF α antibody-producing cell, typically a spleen cell of an animal immunized against either natural or recombinant human TNF, or a peptide fragment of the human TNF α protein sequence. Alternatively, the non-human anti-TNF α antibody-producing cell may be a B lymphocyte obtained from the blood, spleen, lymph nodes or other tissue of an animal immunized with TNF.

The antibody-producing cell contributing the nucleotide sequences encoding the antigen-binding region of the chimeric antibody of the present invention may also be produced by transformation of a non-human, such as a primate, or a human cell. For example, a B lymphocyte which produces anti-TNF antibody may be infected and transformed with a virus such as Epstein-Barr virus to yield an immortal anti-TNF producing cell (Kozbor et al. Immunol. Today 4:72-79 (1983)). Alternatively, the B lymphocyte may be transformed by providing a transforming gene or transforming gene product, as is well-known in the art.

Preferably, the antigen binding region will be of murine origin. In other embodiments, the antigen binding region

may be derived from other animal species, in particular rodents such as rat or hamster.

5 The second fusion partner, which provides the immortalizing function, may be lymphoblastoid cell or a plasmacytoma or myeloma cell, which is not itself an antibody producing cell, but is malignant. Preferred fusion partner cells include the hybridoma SP2/0-Ag14, abbreviated as SP2/0 (ATCC CRL1581) and the myeloma P3X63Ag8 (ATCC TIB9), or its derivatives (see: Hartlow, E. et al., Antibodies: A Laboratory
10 Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988)).

Murine hybridomas which produce mAb specific for human TNF α are formed by the fusion of a mouse fusion partner cell, such as SP2/0, and spleen cells from mice immunized against
15 purified hTNF α , recombinant hTNF α , natural or synthetic TNF peptides, or other biological preparations containing TNF. To immunize the mice, a variety of different conventional protocols may be followed. For example, mice may receive primary and boosting immunizations of TNF.

20 The cell fusions are accomplished by standard procedures well known to those skilled in the field of immunology (Kohler and Milstein, Nature 256:495-497 (1975) and U.S. Patent No. 4,376,110; Hartlow, E. et al., supra; Campbell, A., "Monoclonal Antibody Technology," In: Laboratory Techniques in
25 Biochemistry anti Molecular Biology, Volume 13 (Burdon, R., et al., eds.), Elsevier, Amsterdam (1984); Kennett et al.,

Monoclonal Antibodies (Kennett, et al., eds. pp. 365-367, Plenum Press, NY, 1980); de St. Groth, S.F., et al., J. Immunol. Meth. 35: 1-21 (1980); Galfre, G. et al., Methods Enzymol. 73:3-46 (1981); Goding, J.W. 1987. Monoclonal Antibodies: Principles and Practice. 2nd ed. Academic Press, London, 1987) ;

Fusion partner cell lines and methods for fusing and selecting hybridomas and screening for mAbs are well known in the art (Hartlow, E. et al., supra; Kawamoto, T. et al., Meth. Enzymol 121:266-277 (1986); Kearney, J.F. et al., J. Immunol. 123:1548-1550 (1979); Kilmartin, J.V. et al., J. Cell Biol. 93:576-582 (1982); Kohler, G. et al., Eur. J. Immunol. 6:292-295 (1976); Lane, D.P. et al., J. Immunol. Meth. 47:303-307 (1981); Mueller, U.W. et al., J. Immunol. Meth. 87:193-196 (1986); Pontecorvo, G., Somatic Cell Genet. 1:397-400 (1975); Sharo, J., et al., Proc. Natl. Acad. Sci. USA 76:1420-1424 (1979); Shulman, M. et al., Nature 276:269-270 (1978); Springer, T.A. (ed), Hybridoma Technology in the Biosciences and Medicine, Plenum Press, New York, 1985; and Taggart, R.T. et al., Science 219:1228-1230 (1982)).

The hTNF α -specific murine or chimeric mAb of the present invention may be produced in large quantities by injecting hybridoma or transfectoma cells secreting the antibody into the peritoneal cavity of mice and, after appropriate time, harvesting the ascites fluid which contains a high titer of the mAb, and isolating the mAb therefrom. For such in vivo production of the mAb with a non-murine hybridoma (e.g., rat or

human), hybridoma cells are preferably grown in irradiated or athymic nude mice.

Alternatively, the antibodies may be produced by culturing hybridoma or transfectoma cells in vitro and isolating secreted mAb from the cell culture medium.

Human genes which encode the constant (C) regions of the chimeric antibodies of the present invention were derived from a human fetal liver library. Human C regions genes may be derived from any human cell including those which express and produce human immunoglobulins. The human C_H region can be derived from any of the known classes or isotypes of human H chains, including gamma, μ , α , δ or ϵ , and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions of an antibody, the choice of C_H region will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC). Preferably, the CH region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), or μ (IgM).

The human C_L region can be derived from either human L chain isotype, kappa or lambda.

Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques (Sambrook, J. et al., (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). Human C region genes are readily available from known

clones containing genes representing the two classes of L chains, the five classes of H chains and subclasses thereof. Chimeric antibody fragments, such as F(ab')₂ and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding an H chain portion of an F(ab')₂ fragment would include DNA sequences encoding the CH₁ domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Generally, the chimeric antibodies of the present invention are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a TNF-specific antibody, preferably non-human, and joining these DNA segments to DNA segments encoding human C_H and C_L regions, respectively, to produce chimeric immunoglobulin-encoding genes.

Thus, in a preferred embodiment, a fused gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment encoding at least a part of a human C region.

The DNA encoding the antibody-binding region may be genomic DNA or cDNA. A convenient alternative to the use of chromosomal gene fragments as the source of DNA encoding the murine V region antigen-binding segment is the use of cDNA for the construction of chimeric immunoglobulin genes, as reported by Liu et al. (Proc. Natl. Acad. Sci., USA 84:3439 (1987) and J.

Immunology 139:3521 (1987), which references are hereby incorporated by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired protein. The use of cDNA sequences is advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

Therefore, in an embodiment utilizing cDNA encoding the antibody V region, the method of producing the chimeric antibody involves several steps, outlined below:

1. Isolation of messenger RNA (mRNA) from the cell line producing the monoclonal antibody, cloning and cDNA production therefrom;
2. Preparation of a full length cDNA library from purified mRNA from which the appropriate V region gene segments of the L and H chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C gene segment;
3. Preparation of C region gene segments by cDNA preparation and cloning;
4. Construction of complete H or L chain coding sequences by linkage of the cloned specific V region gene segments to cloned human C region gene, as described above;
5. Expression and production of chimeric L and H chains in selected hosts, including prokaryotic and eukaryotic cells.

One common feature of all immunoglobulin H and L chain genes and their encoded mRNAs is the J region. H and L chain J regions have different sequences, but a high degree of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this method and consensus sequences of H and L chain J regions may be used to design oligonucleotides for use as primers for introducing useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments.

C region cDNA vectors prepared from human cells can be modified by site-directed mutagenesis to place a restriction site at the analogous position in the human sequence. For example, one can clone the complete human kappa chain C (C_k) region and the complete human gamma-1 C region ($C_{\text{gamma-1}}$). In this case, the alternative method based upon genomic C region clones as the source for C region vectors would not allow these genes to be expressed in bacterial systems where enzymes needed to remove intervening sequences are absent. Cloned V region segments are excised and ligated to L or H chain C region vectors. Alternatively, the human $C_{\text{gamma-1}}$ region can be modified by introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule. The coding sequences with linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human C_H or C_L chain sequence having appropriate restriction sites engineered so that any V_H or V_L chain sequence with appropriate cohesive ends can be easily inserted therein. Human C_H or C_L chain sequence-containing vehicles thus serve as intermediates for the expression of any desired complete H or L chain in any appropriate host.

A chimeric mouse-human antibody will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human C_H region; polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

Gene expression elements useful for the expression of cDNA genes include: (a) viral transcription promoters and their

enhancer elements, such as the SV40 early promoter (Okayama, H.
et al., Mol. Cell. Biol. 3:280 (1983)), Rous sarcoma virus LTR
(Gorman, C. et al., Proc. Natl. Acad. Sci., USA 79:6777 (1982)),
and Moloney murine leukemia virus LTR (Grosschedl, R. et al., Cell
5 41:885 (1985)); (b) splice regions and polyadenylation sites
such as those derived from the SV40 late region (Okayama et al.,
supra); and (c) polyadenylation sites such as in SV40 (Okayama
et al., supra).

Immunoglobulin cDNA genes may be expressed as described
10 by Liu et al., supra, and Weidle et al., Gene 51:21 (1987),
using as expression elements the SV40 early promoter and its
enhancer, the mouse immunoglobulin H chain promoter enhancers,
SV40 late region mRNA splicing, rabbit β -globin intervening
sequence, immunoglobulin and rabbit β -globin polyadenylation
15 sites, and SV40 polyadenylation elements. For immunoglobulin
genes comprised of part cDNA, part genomic DNA (Whittle et al.,
Protein Engineering 1:499 (1987)), the transcriptional promoter
is human cytomegalovirus, the promoter enhancers are
cytomegalovirus and mouse/human immunoglobulin, and mRNA splicing
20 and polyadenylation regions are from the native chromosomal
immunoglobulin sequences.

In one embodiment, for expression of cDNA genes in
rodent cells, the transcriptional promoter is a viral LTR
sequence, the transcriptional promoter enhancers are either or
25 both the mouse immunoglobulin heavy chain enhancer and the viral
LTR enhancer, the splice region contains an intron of greater

than 31 bp, and the polyadenylation and transcription termination regions are derived from the native chromosomal sequence corresponding to the immunoglobulin chain being synthesized. In other embodiments, cDNA sequences encoding other proteins are combined with the above-recited expression elements to achieve expression of the proteins in mammalian cells.

Each fused gene is assembled in, or inserted into, an expression vector. Recipient cells capable of expressing the chimeric immunoglobulin chain gene product are then transfected singly with a chimeric H or chimeric L chain-encoding gene, or are co-transfected with a chimeric H and a chimeric L chain gene. The transfected recipient cells are cultured under conditions that permit expression of the incorporated genes and the expressed immunoglobulin chains or intact antibodies or fragments are recovered from the culture.

In one embodiment, the fused genes encoding the chimeric H and L chains, or portions thereof, are assembled in separate expression vectors that are then used to co-transfect a recipient cell.

Each vector may contain two selectable genes, a first selectable gene designed for selection in a bacterial system and a second selectable gene designed for selection in a eukaryotic system, wherein each vector has a different pair of genes. This strategy results in vectors which first direct the production, and permit amplification, of the fused genes in a bacterial system. The genes so produced and amplified in a bacterial host

are subsequently used to co-transfect a eukaryotic cell, and allow selection of a co-transfected cell carrying the desired transfected genes.

Examples of selectable genes for use in a bacterial system are the gene that confers resistance to ampicillin and the gene that confers resistance to chloramphenicol. Preferred selectable genes for use in eukaryotic transfectants include the xanthine guanine phosphoribosyl transferase gene (designated gpt) and the phosphotransferase gene from Tn5 (designated neo). Selection of cells expressing gpt is based on the fact that the enzyme encoded by this gene utilizes xanthine as a substrate for purine nucleotide synthesis, whereas the analogous endogenous enzyme cannot. In a medium containing (1) mycophenolic acid, which blocks the conversion of inosine monophosphate to xanthine monophosphate, and (2) xanthine, only cells expressing the gpt gene can survive. The product of the neo blocks the inhibition of protein synthesis by the antibiotic G418 and other antibiotics of the neomycin class.

The two selection procedures can be used simultaneously or sequentially to select for the expression of immunoglobulin chain genes introduced on two different DNA vectors into a eukaryotic cell. It is not necessary to include different selectable markers for eukaryotic cells; an H and an L chain vector, each containing the same selectable marker can be co-transfected. After selection of the appropriately resistant

cells, the majority of the clones will contain integrated copies of both H and L chain vectors.

Alternatively, the fused genes encoding the chimeric H and L chains can be assembled on the same expression vector.

5 For transfection of the expression vectors and production of the chimeric antibody, the preferred recipient cell line is a myeloma cell. Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for glycosylation of the
10 immunoglobulin. A particularly preferred recipient cell is the Ig-non-producing myeloma cell SP2/0 (ATCC #CRL 8287). SP2/0 cells produce only immunoglobulin encoded by the transfected genes. Myeloma cells can be grown in culture or in the peritoneal cavity of a mouse, where secreted immunoglobulin can
15 be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of human or non-human origin, hybridoma cells of human or non-human origin, or interspecies heterohybridoma cells.

The expression vector carrying a chimeric antibody
20 construct of the present invention may be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE)
25 dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile bombardment (Johnston et al.,

Science 240:1538 (1988)). A preferred way of introducing DNA into lymphoid cells is by electroporation (Potter et al., Proc. Natl. Acad. Sci. USA 81:7161 (1984); Yoshikawa, K. et al., Jpn. J. Cancer Res. 77:1122-1133). In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated. Typically, after transfection, cells are allowed to recover in complete medium for about 24 hours, and are then seeded in 96-well culture plates in the presence of the selective medium. G418 selection is performed using about 0.4 to 0.8 mg/ml G418. Mycophenolic acid selection utilizes about 6µg/ml plus about 0.25 mg/ml xanthine. The electroporation technique is expected to yield transfection frequencies of about 10^{-5} to about 10^{-4} for Sp2/0 cells. In the protoplast fusion method, lysozyme is used to strip cell walls from catarrhal harboring the recombinant plasmid containing the chimeric antibody gene. The resulting spheroplasts are fused with myeloma cells with polyethylene glycol.

The chimeric immunoglobulin genes of the present invention can also be expressed in nonlymphoid mammalian cells or in other eukaryotic cells, such as yeast, or in prokaryotic cells, in particular bacteria.

Yeast provides substantial advantages over bacteria for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number

plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides) (Hitzman, et al., 11th International Conference on Yeast, Genetics and Molecular Biology, Montpellier, France, September 13-17, 1982).

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of chimeric H and L chain proteins and assembled chimeric antibodies. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. A number of approaches may be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast (see Glover, D.M., ed., DNA Cloning, Vol. II, pp45-66, IRL Press, 1985).

Bacterial strains may also be utilized as hosts for the production of antibody molecules or antibody fragments described by this invention, E. coli K12 strains such as E. coli W3110 (ATCC 27325), and other enterobacteria such as Salmonella

antibodies. As discussed above, it is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H_2L_2 antibodies. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells may be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing H_2L_2 molecules via either route could be transfected with plasmids encoding additional copies of H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H_2L_2 antibody molecules or enhanced stability of the transfected cell lines.

In addition to monoclonal or chimeric anti-TNF antibodies, the present invention is also directed to an anti-idiotypic (anti-Id) antibody specific for the anti-TNF antibody of the invention. An anti-Id antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding region of another antibody. The antibody specific for TNF is termed the idiotypic or Id antibody. The anti-Id can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the

typhimurium or Serratia marcescens, and various Pseudomonas species may be used.

Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches may be taken for evaluating the expression plasmids for the production of chimeric antibodies or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, D.M., ed., DNA Cloning, Vol. I, IRL Press, 1985).

Preferred hosts are mammalian cells, grown in vitro or in vivo. Mammalian cells provide post-translational modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which may be useful as hosts for the production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO-K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned H and L chain genes in mammalian cells (see Glover, D.M., ed., DNA Cloning, Vol. II, pp143-238, IRL Press, 1985).

Different approaches can be followed to obtain complete H₂L₂

Id antibody with the Id antibody or the antigen-binding region thereof. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody and produce an anti-Id antibody. The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original antibody which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against TNF according to the present invention may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice can be used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a TNF epitope.

The antibodies, fragments and derivatives of the present invention are useful for treating a subject having a disease or condition associated with levels of a substance reactive with an anti-TNF antibody, in particular TNF, in excess of the levels present in a normal healthy subject. Such diseases include, but are not limited to, sepsis syndrome, including cachexia, circulatory collapse and shock resulting from acute or

chronic bacterial infection, acute and chronic parasitic or infectious processes, including bacterial, viral and fungal infections, acute and chronic immune and autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, alcohol-induced hepatitis, chronic inflammatory diseases such as sarcoidosis and Crohn's disease, vascular inflammatory diseases such as disseminated intravascular coagulation, graft-versus-host disease, Kawasaki's disease and malignant diseases involving TNF-secreting tumors.

Such treatment comprises parenterally administering a single or multiple doses of the antibody, fragment or derivative. Preferred for human pharmaceutical use are high affinity potent hTNF α -neutralizing chimeric antibodies of this invention.

Monoclonal antibodies may be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. In the case of the antibodies of this invention, the primary focus is the ability to reach and bind with TNF released by monocytes and macrophages. Because proteins are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, intramuscular, would ordinarily be used to optimize absorption.

Monoclonal antibodies may be administered either as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on

the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Usually a daily dosage of active ingredient can be about 0.1 to 100 milligrams per kilogram of body weight. Ordinarily 0.5 to 50, and preferably 1 to 10 milligrams per kilogram per day given in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

Dosage forms (composition) suitable for internal administration generally contain from about 1 milligram to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

For parenteral administration, the antibody can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The vehicle or lyophilized powder may contain additives

that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

5 Suitable pharmaceutical carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field.

For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

10 The antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) against cells having TNF associated with their surface. For these activities, either an
15 endogenous source or an exogenous source of effector cells (for ADCC) or complement components (for CDC) can be utilized. The chimeric antibodies of this invention, their fragments, and derivatives can be used therapeutically as immunoconjugates (see for review: Dillman, R.O., Ann. Int. Med. 111:592-603 (1989)).
20 They can be coupled to cytotoxic proteins, including, but not limited to Ricin-A, Pseudomonas toxin, Diphtheria toxin, and TNF. Toxins conjugated to antibodies or other ligands, are known in the art (see, for example, Olsnes, S. et al., Immunol. Today 10:291-295 (1989)). Plant and bacterial toxins typically kill
25 cells by disrupting the protein synthetic machinery.

The antibodies of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, cytotoxic agents and drugs. Examples of radionuclides which can be coupled to antibodies and delivered in vivo to sites of antigen include ^{212}Bi , ^{131}I , ^{186}Re , and ^{90}Y , which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to antibodies and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A.G., et al., Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed., Macmillan Publishing Co., 1985.

The antibodies of this invention maybe advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

The antibodies, fragments or derivatives of this invention may also be used in combination with TNF therapy to block undesired side effects of TNF. Recent approaches to cancer

therapy have included direct administration of TNF to cancer patients or immunotherapy of cancer patients with lymphokine activated killer (LAK) cells (Rosenberg et al., New Eng. J. Med. 313:1485-1492 (1985)) or tumor infiltrating lymphocytes (TIL)

5 (Kurnick et al., (Clin. Immunol. Immunopath. 38:367-380 (1986);

Kradin et al., Cancer Immunol. Immunother. 24:76-85 (1987);

Kradin et al., Transplant. Proc. 20:336-338 (1988)). Trials are

currently underway using modified LAK cells or TIL which have been transfected with the TNF gene to produce large amounts of

10 TNF. Such therapeutic approaches are likely to be associated with a number of undesired side effects caused by the pleiotropic actions of TNF which were described above. According to the present invention, these side effects can be reduced by

concurrent treatment of a subject receiving TNF or cells

15 producing large amounts of TIL with the antibodies, fragments or derivatives of the present invention. Effective doses are as

described above. The dose level will require adjustment

according to the dose of TNF or TNF-producing cells administered, in order to block side effects without blocking the main anti-

20 tumor effect of TNF. One of ordinary skill in the art will know how to determine such doses without undue experimentation.

The chimeric antibodies, fragments, or derivatives of this invention, attached to a solid support, can be used to remove TNF from fluids or tissue or cell extracts. In a

25 preferred embodiment, they are used to remove TNF from blood or blood plasma products. In another preferred embodiment, the

chimeric antibodies are advantageously used in extracorporeal immunoadsorbent devices, which are known in the art (see, for example, Seminars in Hematology, Vol. 26 (2 Suppl. 1) (1989)). Patient blood or other body fluid is exposed to the attached antibody, resulting in partial or complete removal of circulating TNF (free or in immune complexes), following which the fluid is returned to the body. This immunoadsorption can be implemented in a continuous flow arrangement, with or without interposing a cell centrifugation step. See, for example, Terman, D.S. et al., J. Immunol. 117:1971-1975 (1976).

The present invention also provides the above antibodies, fragments and derivatives, detectably labeled, as described below, for use in diagnostic methods for detecting TNF α in patients known to be or suspected of having a TNF α -mediated condition.

The antibodies of the present invention are useful for immunoassays which detect or quantitate TNF, or anti-TNF antibodies, in a sample. An immunoassay for TNF typically comprises incubating a biological sample in the presence of a detectably labeled high affinity antibody of the present invention capable of selectively binding to TNF, and detecting the labeled antibody which is bound in a sample. Various clinical immunoassay procedures are described in Immunoassays for the 80's, A. Voller et al., eds., University Park, 1981.

Thus, in this aspect of the invention, the antibody or a biological sample may be added to nitrocellulose, or other

solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled TNF-specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support may then be detected by conventional means.

By "solid phase support" or "carrier" is intended any support capable of binding antigen or antibodies. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to TNF or an anti-TNF antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-TNF antibody may be determined according to well known methods.

Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the TNF-specific antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA), or enzyme-linked immunosorbent assay (ELISA). This enzyme, when subsequently exposed to its substrate, will react with the substrate generating a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the TNF-specific antibodies of the present invention include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

By radioactively labeling the TNF-specific antibodies, it is possible to detect TNF through the use of a radioimmunoassay (RIA) (see, for example, Work, T.S., et al., Laboratory Techniques and Biochemistry in Molecular Biology, North Holland Publishing Company, N.Y. (1978). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the

present invention are: 3_H , 125_I , 131_I , 35_S , 14_C , and, preferably, 125_I .

It is also possible to label the TNF-specific antibodies with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthalaldehyde and fluorescamine.

The TNF-specific antibodies can also be detectably labeled using fluorescence-emitting metals such as 152_{Eu} , or others of the lanthanide series. These metals can be attached to the TNF-specific antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The TNF-specific antibodies also can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the TNF-specific antibody, fragment or derivative of the present invention. Bioluminescence is a type of

chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important
5 bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Detection of the TNF-specific antibody, fragment or derivative may be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter,
10 or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme. Detection may also be accomplished by visual
15 comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

For the purposes of the present invention, the TNF which is detected by the above assays may be present in a biological sample. Any sample containing TNF can be used. Preferably, the sample is a biological fluid such as, for
20 example, blood, serum, lymph, urine, inflammatory exudate, cerebrospinal fluid, amniotic fluid, a tissue extract or homogenate, and the like. However, the invention is not limited to assays using only these samples, it being possible for one of ordinary skill in the art to determine suitable conditions which
25 allow the use of other samples.

In situ detection may be accomplished by removing a histological specimen from a patient, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably
5 provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of TNF but also the distribution of TNF in the examined tissue.

Using the present invention, those of ordinary skill will readily
10 perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

The antibody, fragment or derivative of the present invention may be adapted for utilization in an immunometric
15 assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support that is insoluble in the fluid being tested and a quantity of detectably labeled soluble antibody is added to permit detection and/or
20 quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the
25 TNF from the sample by formation of a binary solid phase antibody-TNF complex. After a suitable incubation period, the

solid support is washed to remove the residue of the fluid sample, including unreacted TNF, if any, and then contacted with the solution containing a known quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the TNF bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay may be a simple "yes/no" assay to determine whether TNF is present or may be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of TNF. Such "two-site" or "sandwich" assays are described by Wide (Radioimmune Assay Method, Kirkham, ed., E. & S. Livingstone, Edinburgh, 1970, pp. 199-206).

Other type of "sandwich" assays, which may also be useful with TNF, are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step wherein the antibody bound to the solid support and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the

addition of unlabeled antibody bound to a solid support after a suitable incubation period, is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays. In one embodiment, a combination of antibodies of the present invention specific for separate epitopes may be used to construct a sensitive three-site immunoradiometric assay.

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE I

Production of A2, a Mouse Anti-Human TNF mAb

To facilitate clinical study of TNF mAb a high-affinity potent neutralizing mouse anti-human TNF IgG1 mAb designated A2 was produced. Female BALB/c mice, 10 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME). Forty μ g of purified E-coli-derived recombinant human TNF (rhTNF) emulsified with an equal volume of complete Freund's adjuvant (obtained from

Difco Laboratories) in 0.4 ml was injected subcutaneously and intraperitoneally (i.p.) into a mouse. One week later, an injection of 5 µg of rhTNF in incomplete Freund's adjuvant was given i.p. followed by four consecutive i.p. injections of 10 µg of TNF without adjuvant. Eight weeks after the last injection, the mouse was boosted i.p. with 10 µg of TNF.

Four days later, the mouse was sacrificed, the spleen was obtained and a spleen cell suspension was prepared. Spleen cells were fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37°C for 6 hours, the fused cells were distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4 SP2/0 cells per well. Feeder cells, in the form of 5×10^4 normal BALB/c spleen cells, were added to each well.

The growth medium used consisted of RPMI-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim).

A solid-phase radioimmunoassay (RIA) was employed for screening supernatants for the presence of mAbs specific for rhTNFα. This assay is described in Example II, below. The background binding in this assay was about 500 cpm. A

supernatant was considered positive if it yielded binding of 2000 cpm or higher.

Of 322 supernatants screened, 25 were positive by RIA. Of these 25, the one with the highest binding (4800 cpm) was designated A2. Positive wells were subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, A2 was found to be the only positive clone showing potent neutralizing activity. Thus, the hybridoma line A2 was selected. This line was maintained in RPMI-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

EXAMPLE II

Characterization of mAb A2

A. Radioimmunoassays

E. coli-derived rhTNF was diluted to 1 µg/ml in BCB buffer, pH 9.6, and 0.1 ml of the solution was added to each assay well. After incubation at 4°C overnight, the wells were washed briefly with BCB, then sealed with 1% bovine serum albumin (BSA) in BCB at 37°C for 1 hr. The wells were then washed 3 times with PBS containing 0.05% Tween-20 (PBS-Tween), and 70 µl diluted A2 ascites fluid was added to each well. The wells were incubated for 2 hr at 37°C, and washed 3 times with PBS-Tween. Thereafter, approximately 50,000 cpm of ¹²⁵I-labeled F(ab')²

fragment of sheep anti-mouse Ig antibodies in 50 μ l of PBS-Tween containing 1% BSA was added to each well, and the wells were incubated for an additional 2 hr at 37°C. The wells were washed 4 times with PBS-Tween, cut out and counted individually.

5 Results of two determinations are shown in Figure 1.

rhTNF at 5 μ g/ml in PBS was heated to 60°C. At various time points, aliquots of the heat-treated TNF preparation were quickly cooled to 4°C; diluted 5-fold in BCB, and used to coat the RIA microplate wells. The RIA was carried out exactly
10 as described above. Results from two determinations are shown in Figure 2. As incubation at 60°C substantially reduced the biological activity of hTNF α , this experiment shows mAb A2 fails to bind heat-inactivated human TNF α .

B. Neutralization Assays

15 Samples of A2 and cA2 were purified by protein A affinity chromatography from hybridoma tissue culture supernatants of cell lines designated C134A and C168A (described above), respectively, and diafiltered in phosphate buffered saline pH 7.2 (PBS).

20 The toxic effect of TNF on certain tumor cell lines has been adapted as an in vitro measure of TNF levels in laboratory samples and biological fluids. The assay method of Feinman et al., J Immunol 138:635-640 (1987), as modified by Aderka et al., J. Immunol. 143:3517-3523 (1989), employing the TNF-sensitive
25 target cell A673 (a human rhabdomyosarcoma cell line), was used to investigate the ability of A2 to neutralize TNF toxicity.

Cultured human A673/6 cells were incubated with 40 pg/ml of natural (Genzyme, Boston, MA) or recombinant (Suntory, Osaka, Japan) human TNF α with varying concentrations of mAb A2 in the presence of 20 μ g/ml cycloheximide at 39°C overnight.

5 Controls included medium alone or medium + TNF in each well. Cell death was measured by staining with naphthol blue-black, and the results read spectrophotometrically at 630 nm. Absorbance at this wave length correlates with the number of live cells present.

10 It was found that A2 neutralized the cytotoxic effect of both natural and rhTNF in a dose-dependent manner (Figure 3).

In another experiment, the specificity of this neutralizing activity was tested. A673/6 cells were seeded at 3×10^4 cells/well 20 hr before the TNF bioassay. Two-fold serial
15 dilutions of rhTNF, E. coli-derived recombinant human lymphotoxin (TNF β), and E. coli-derived recombinant murine TNF were prepared. The A2 hybridoma supernatant was added to an equal volume of the diluted TNF preparations, and the mixtures were incubated at room temperature for 30 min. Aliquots of 0.1 ml were transferred to
20 the wells containing A673/6 cells, 20 μ g/ml of cycloheximide was added, and the cells were incubated at 39°C overnight. The cells were then fixed and stained for evaluation of cytotoxicity. The results indicate that mAb A2 specifically neutralized the cytotoxicity of rhTNF α , whereas it had no effect on human
25 lymphotoxin (TNF β) (Figure 4) or murine TNF (Figure 5).

Experiments were next performed to analyze the cross-reactivity of mAb A2 with TNF derived from non-human primates. Monocytes isolated from B514 (baboon), J91 (cynomolgus) and RH383 (rhesus) blood by Ficoll gradient centrifugation and adherence, were incubated at 1×10^5 cells/well in RPMI 1640 medium with 5% FBS and 2 $\mu\text{g/ml}$ of *E. coli* LPS for 3 or 16 hr at 37°C to induce TNF production. Supernatants from duplicate wells were pooled and stored at 4°C for less than 20 hr until the TNF bioassay was performed, as described above, using A673/6 cells. Two-fold dilutions of the culture supernatants were mixed with either medium or purified mAb A2 at a final concentration of 1 $\mu\text{g/ml}$, incubated at room temperature for 30 min and aliquots transferred to the indicator cells. The results showed that mAb A2 failed to significantly neutralize the cytotoxic activity of TNF produced by baboon, cynomolgus and rhesus monkey monocytes.

A further experiment was conducted with chimpanzee TNF. Monocytes isolated from CH563 (chimpanzee) blood were incubated as described above to generate TNF-containing supernatants. The ability of 10 $\mu\text{g/ml}$ of mAb A2 to neutralize the bioactivity of these supernatants was assayed as above. Human TNF was used as a positive control. Results, shown in Figure 6, indicate that mAb A2 had potent neutralizing activity for chimpanzee TNF, similar to that for human TNF (Figure 7).

The neutralizing activity of mAb A2 was compared with three other murine mAbs specific for human TNF, termed TNF-1, TNF-2 and TNF-3, and a control mAb. Two-fold serial dilutions

of purified mAbs were mixed with rhTNF (40 pg/ml), incubated at room temperature for 30 min, and aliquots tested for TNF bioactivity as above. It was found that mAbs TNF-1, TNF-2 and TNF-3 each had a similar moderate degree of neutralizing activity. In contrast, mAb A2 had much more potent neutralizing activity.

EXAMPLE III

General Strategy for Cloning Antibody V and C Genes

The strategy for cloning the V regions for the H and L chain genes from the hybridoma A2, which secretes the anti-TNF antibody described above, was based upon the linkage in the genome between the V region and the corresponding J (joining) region for functionally rearranged (and expressed) Ig genes. J region DNA probes can be used to screen genomic libraries to isolate DNA linked to the J regions. Although DNA in the germline configuration (i.e., unrearranged) would also hybridize to J probes, this DNA would not be linked to a Ig V region sequence and can be identified by restriction enzyme analysis of the isolated clones.

The cloning utilized herein was to isolate V regions from rearranged H and L chain genes using J_H and J_k probes. These clones were tested to see if their sequences were expressed in the A2 hybridoma by Northern analysis. Those clones that contained expressed sequence were cloned into

expression vectors containing human C regions and transfected into mouse myeloma cells to determine if an antibody was produced. The antibody from producing cells was then tested for binding specificity and functionally compared to the A2 murine antibody.

EXAMPLE IV

Construction of a L Chain Genomic Library

To isolate the L chain V region gene from the A2 hybridoma, a size-selected genomic library was constructed using the phage lambda vector charon 27. High molecular weight DNA was isolated from A2 hybridoma cells and digested to completion with restriction endonuclease HindIII. The DNA was then fractionated on a 0.8% agarose gel and the DNA fragments of three different size ranges of approximately 3 kb, 4 kb and 6 kb were isolated from the gel by electroelution. The size ranges for library construction were chosen based upon the size of HindIII fragments that hybridized on a southern blot with the J_k probe. After phenol/chloroform extraction and ethanol precipitation, the DNA fragments from each size class were ligated with lambda charon 27 arms and packaged into phage particles in vitro using Gigapack Gold from Stratagene (LaJolla, CA).

These libraries were screened directly at a density of approximately 20,000 plaques per 150 mm petri dish using a ³²P-labeled J_k probe. The mouse L chain J_k probe was a 2.7 kb HindIII fragment containing all five J_k segments. The probe was

EXAMPLE VI

Cloning of the TNF-Specific V gene regions

Several positive clones were isolated from the H and L chain libraries after screening approximately 10^6 plaques from each library using the J_H and J_k probes, respectively. Following plaque purification, bacteriophage DNA was isolated for each positive clone, digested with either EcoRI (H chain clones) or HindIII (L chain clones) and fractionated on 1% agarose gels. The DNA was transferred to nitrocellulose and the blots were hybridized with the J_H or the J_k probe.

Several H chain clones were obtained that contained 7.5 kb EcoRI DNA fragments that hybridized to the J_H probe. For the light chain libraries, several clones from each of the three size-selected libraries were isolated that contained HindIII fragments that hybridize to the J_k probe. For the L chain, several independently derived HindIII fragments of 2.9 kb from the 2 kb library hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA (see Example VII). In addition, several HindIII fragments derived from the 4 kb library hybridized both to the A2 mRNA and the fusion partner mRNA. A 5.7 kb HindIII fragment from the 6 kb library did not hybridize to either RNA.

The observed lengths of hybridizing A2 mRNA were the correct sizes for H and L chain mRNA, respectively. Because the RNA expression was restricted to the A2 hybridoma, it was assumed that the 7.5 kb H chain fragments and the 2.9 kb L chain

fragments contained the correct V region sequences from A2. One example of each type was chosen for further study. The important functional test is the demonstration that these V region sequences, when combined with appropriate C region sequences, are capable of directing the synthesis of an antibody with a specificity and affinity similar to that of the murine A2 antibody.

The 7.5 kb H chain fragment and the 2.9 kb L chain fragment were subcloned into plasmid vectors that allow expression of the chimeric mouse/human proteins in murine myeloma cells (see Examples VIII and IX). These plasmids were cotransfected into SP2/0 cells to ascertain if intact antibody molecules were secreted, and if so, if they were of the correct specificity and affinity. Control transfections were also performed pairing the putative anti-TNF H chain with an irrelevant, but expressed, L chain; the putative anti-TNF L chain was also paired with an irrelevant, but expressed, H chain. The results indicated that the 7.5 kb H chain fragment could be expressed, whereas the 2.9 kb L chain fragment could not. This was confirmed by DNA sequence analysis that suggested portions of the coding region were not in the proper amino acid reading frame when compared to other known L chain amino acid sequences.

Because the 2.9 kb HindIII fragment appeared not to contain a functional V gene, the 4.0 kb and 5.7 kb HindIII fragments isolated from L chain libraries were cloned into expression vectors and tested for expression of chimeric antibody

after cotransfection with the 7.5 kb H chain. The 5.7 kb HindIII fragment was incapable of supporting antibody expression, whereas the 4.0 kb HindIII fragment did support antibody expression. The antibody resulting from the cotransfection of the 7.5 kb putative H chain V region and the 4.0 kb L chain V region was purified, tested in solid phase TNF binding assay, and found to be inactive. It was concluded that the V region contained on the 4.0 kb HindIII fragment was not the correct anti-TNF V regions, but was contributed to the hybridoma by the fusion partner. This was subsequently confirmed by sequence analysis of cDNA derived from the A2 hybridoma and from the fusion partner.

Other independently derived L chain clones containing 2.9 kb HindIII fragments that hybridized with A2 mRNA were characterized in more detail. Although the restriction maps were similar, the clones fell into two classes with respect to the presence or absence of an AccI enzyme site. The original (non-functional) 2.9 kb fragment (designated clone 8.3) was missing an AccI site present in some other clones (represented by clone 4.3). The DNA sequence of clone 4.3 was extremely similar to clone 8.3, but contained a single amino acid reading frame with close homology to known L chains, unlike clone 8.3. The 2.9 kb HindIII fragment from clone 4.3 was subcloned into the L chain expression vector and cotransfected with the putative anti-TNF H chain into SP2/0 cells. An antibody was synthesized, purified and tested in the solid phase TNF binding assay. This antibody

bound to TNF, and therefore, the clone 4.3 L chain V region was assumed to be the correct one.

The A2 murine hybridoma has been shown to contain at least four rearranged L chain V region genes. At least two of these are expressed as proteins: clone 4.3 (the correct anti-TNF L chain gene) and the gene contained in the 4.0 kb HindIII fragment (contributed by the fusion partner). The expression of two L chains implies that the resulting antibody secreted from the murine hybridoma is actually a mixture of antibodies, some using the correct L chain, some using the incorrect L chain, and some using one of each. The presence of two different L chains in the murine A2 antibody has been confirmed by SDS gel and N-terminal protein sequence analysis of the purified antibody. Because construction of the chimeric A2 antibody involves cloning the individual H and L chain genes and expressing them in a non-producing cell line, the resulting antibody will have only the correct L chain and therefore should be a more potent antibody (see Examples X, XI and XII).

EXAMPLE VII

Northern Analysis of Cloned DNA

Cloned DNA corresponding to the authentic H and L chain V regions from the A2 hybridoma would be expected to hybridize to A2 mRNA. Non-functional DNA rearrangements at either the H or L chain genetic loci should not be expressed.

Ten µg total cellular RNA was subjected to electrophoresis on 1% agarose/formaldehyde gels (Sambrook et al, supra) and transferred to nitrocellulose. Blots were hybridized with random primed DNA probes in 50% formamide, 2x Denhardt's solution, 5x SSC, and 200 µg/ml denatured salmon sperm DNA at 42°C for 10 hours. Final wash conditions were 0.5 x SSC, 0.1% SDS at 65°C.

The subcloned DNA fragments were labeled with ³²P by random priming and hybridized to Northern blots containing total RNA derived from A2 cells or from cells of SP2/0, the fusion partner parent of A2. The 7.5 EcoRI H chain fragment hybridized with a 2 kb mRNA from A2, but not with SP2/0 mRNA. Similarly, the 2.9 kb L chain HindIII fragment (clone 4.3) hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA. The observed lengths of A2 mRNA hybridizing were the correct sizes for H and L chain mRNA, respectively, confirming that the V region sequences on these DNA fragments are expressed in A2 hybridoma cells.

EXAMPLE VIII

Construction of Expression Vectors

The putative L (clone 4.3) and H chain V genes described above were joined to human kappa and gamma constant region genes in expression vectors. The 7.5 kb EcoRI fragment corresponding to the putative V_H region gene from A2 was cloned

into an expression vector containing the human C_{gamma1} gene and the Ecoipt gene to yield the plasmid designated pA2HGlapgpt (see Figure 8).

The 2.9 kb putative V_L fragment from clone 4.3 was cloned into a vector containing the human kappa C_k gene and the Ecoipt gene to allow selection in mammalian cells. The resulting plasmid was designated pA2HuKapgpt (See Figure 8).

EXAMPLE IX

Expression of Chimeric Antibody Genes

To express the chimeric H and L chain genes, the expression plasmids were transfected into cells of the non-producing mouse myeloma cell line, SP2/0. Plasmid DNA to be transfected was purified by centrifuging to equilibrium in ethidium bromide/cesium chloride gradients twice. Plasmid DNA (10-50 µg) was added to 10⁷ SP2/0 cells in medium containing Hank's salts, and the mixture was placed in a BioRad electroporation apparatus. Electroporation was performed at 20 volts, following which the cells were plated in 96 well microtiter plates.

Mycophenolic acid selection was applied after 24 hours and drug resistant colonies were identified after 1-2 weeks. Resistant colonies were expanded to stable cell lines and tissue culture supernatant from these cell lines was tested for antibody using an ELISA assay with goat anti-human IgG Fc antibody and

goat anti-human H+L conjugated with alkaline phosphatase
(obtained from Jackson Laboratories).

The chimeric A2 antibody was purified from tissue
culture supernatant by Protein A- Sepharose chromatography. The
5 supernatant was adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and
loaded on a Protein A-Sepharose column equilibrated in the same
buffer. The IgG was eluted with 0.1M citrate, pH 3.5,
neutralized with 1M Tris, and dialyzed into phosphate buffered
saline (PBS).

10 The purified chimeric antibody was evaluated for its
binding and neutralizing activity.

EXAMPLE X

Specificity of Chimeric A2 Antibody

Since the antigen binding domain of cA2 was derived
15 from murine A2, these mAbs would be expected to compete for the
same binding site on TNF. Fixed concentrations of chimeric A2
and murine mAb A2 were incubated with increasing concentrations
of murine and chimeric A2 competitor, respectively, in a 96-well
microtiter plate coated with rhTNF (Dainippon, Osaka, Japan).
20 Alkaline-phosphatase conjugated anti-human immunoglobulin and
anti-mouse immunoglobulin second antibodies were used to detect
the level of binding of chimeric and murine A2, respectively.
Cross-competition for TNF antigen was observed in this solid-

phase ELISA format (Figure 9). This finding is consistent with the expected identical epitope specificity of cA2 and murine A2.

The affinity constant for binding of mouse mAb A2 and cA2 to rhTNF α was determined by Scatchard analysis (see, for example, Scatchard, G., Ann. N.Y. Acad. Sci. 51:660 (1949)).

The results are shown in Figure 10. This analysis involved measuring the direct binding of ^{125}I labelled cA2 to immobilized rhTNF α in a 96-well plate. The antibodies were each labelled to a specific activity of about 9.7 $\mu\text{Ci}/\mu\text{g}$ by the iodogen method. An affinity constant (K_a) of 0.5×10^9 liters/mole was calculated for the mouse mAb A2. Unexpectedly, the chimeric A2 antibody had a higher affinity, with a K_a of 1.8×10^9 liters/mole. Thus, the chimeric anti-TNF α antibody of the present invention was shown to exhibit a significantly higher affinity of binding to human TNF α than did the parental murine A2 mAb. This finding was surprising, since chimeric antibodies would be expected to have affinities that are equal to or less than that of the parent mAb.

Such high affinity anti-TNF antibodies, having affinities of binding to TNF α of at least $1 \times 10^8 \text{ M}^{-1}$, more preferably at least $1 \times 10^9 \text{ M}^{-1}$ (expressed as K_a) are preferred for immunoassays which detect very low levels of TNF in biological fluids. In addition, anti-TNF antibodies having such high affinities are preferred for therapy of TNF- α -mediated conditions or disease states.

The specificity of cA2 for TNF was confirmed by testing for cross-neutralization of human lymphotoxin (TNF- β).

Lymphotoxin shares some sequence homology and certain biological activities, for example, tumor cell cytotoxicity, with TNF

5 (Pennica, D. et al., Nature 312:724-729 (1984)). Cultured human A673 cells were incubated with increasing concentrations of human lymphotoxin (Genentech, San Francisco, CA) with or without 4 μ g/ml chimeric A2 in the presence of 20 μ g/ml cycloheximide at 39°C overnight. Cell death was measured by vital staining with
10 naphthol blue-black, as above. The results indicated that cA2 was ineffective at neutralizing human lymphotoxin, confirming the TNF α -specificity of the chimeric antibody.

The ability of A2 or cA2 to react with TNF from different animal species was also evaluated. As mentioned
15 earlier, there are multiple epitopes on human TNF to which neutralizing mAbs will bind (Moller, A. et al., supra). Human TNF has bioactivity in a wide range of host animal species. However, certain neutralizing epitopes on human TNF are conserved amongst different animal species and others appear to be
20 restricted to humans and chimpanzees.

Neutralization experiments utilized endotoxin-activated cell supernatants from freshly isolated human, chimpanzee, rhesus and cynomolgus monkey, baboon, pig, dog, rabbit, or rat monocytes as the TNF source. As discussed above, murine mAb A2 neutralized
25 activity of only human and chimpanzee TNF, and had no effect on TNF derived from other primates and lower animals. A2 also did

not neutralize the cytotoxic effect of recombinant mouse TNF. Thus, the epitope recognized by A2 is one shared by human and chimpanzee TNF α . Chimeric A2 was also tested in this manner for cross-reactivity with monocyte-derived TNF from rat, rabbit, dog and pig, as well as with purified recombinant mouse TNF α , and natural and recombinant human TNF α . Chimeric A2 only neutralized natural and recombinant human TNF α . Therefore, cA2 appears to share species specificity with murine A2.

EXAMPLE XI

In Vitro Activity and Neutralization Efficacy of cA2 Antibody

Both the murine and chimeric anti-TNF α antibodies, A2 and cA2 have potent TNF-neutralizing activity. In the TNF cytotoxicity assay described above, murine A2, at a concentration of about 125 ng/ml completely neutralized the biological activity of a 40 pg/ml challenge of rhTNF α . Two separate determinations of neutralizing potency, expressed as the 50% Inhibitory Dose (ID₅₀) were determined to be 15.9 ± 1.01 and 17.9 ± 1.6 ng/ml (Mean \pm Std error). Thus the mAb A2 has an ID₅₀ of about 17 ng/ml.

In this same experimental system, three other murine anti-TNF α antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF were found to have ID₅₀ values of 1-2 orders of magnitude greater, and thus were significantly less potent in neutralization than A2.

The ability of cA2 to neutralize human TNF α bioactivity in vitro was tested using the bioassay system described above.

Cultured A673 cells were incubated with 40 pg/ml natural (Genzyme, Boston, MA) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death was measured by vital staining. As expected based upon the above results with the A2 mouse mAb, cA2 also neutralized both natural and rhTNF in a dose-dependent manner in the cytotoxicity assay (Figure 11). In this assay format, levels of cA2 as low as 125 ng/ml completely abolished the toxic activity of TNF. Upon repeated analysis, the cA2 exhibited greater TNF-neutralizing activity than did the parent murine A2 mAb. Such neutralizing potency, at antibody levels below 1 μ g/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent neutralizing anti-TNF antibodies, in particular the chimeric antibody, are preferred for therapeutic use in TNF α -mediated diseases or conditions.

As mentioned above, TNF induces cellular secretion of IL-6. Furthermore, there is evidence that IL-6 is involved in the pathophysiology of sepsis, although the precise role of IL-6 in that syndrome is unclear (Fong, Y. et al., J Exp Med 170:1627-1633 (1989); Starnes Jr., H.F. et al., J Immunol 145:4185-4191 (1990)). The ability of cA2 to neutralize TNF-induced IL-6 secretion was evaluated using cultured human diploid FS-4 fibroblasts. The results in Table 1 show that cA2 was effective in blocking IL-6 secretion in cells that had been incubated

overnight with TNF. TNF-induced IL-6 secretion was not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

TABLE 1

IN VITRO NEUTRALIZATION OF TNF-INDUCED IL-6 SECRETION

Antibody	TNF Concentration (ng/ml)			
	0	0.3	1.5	7.5
None	<0.20	1.36	2.00	2.56
Control mAb	<0.20	1.60	1.96	2.16
cA2	<0.20	<0.20	<0.20	0.30

Values represent mean concentrations of IL-6 of duplicate wells, in ng/ml. RhTNF (Suntory, Osaka, Japan), with or without 4 µg/ml antibody, was added to cultures of FS-4 fibroblasts and after 18 h, the supernatant was assayed for IL-6 using the Quantikine Human IL-6 Immunoassay (from R&D Systems, Minneapolis, MN). Control mAb = chimeric mouse/human IgG1 anti-platelet mAb (7E3).

The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of disease pathophysiology. In particular, this may be associated with the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore, the ability of cA2 to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) was evaluated. TNF stimulation of procoagulant activity was determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for

4 hours and analyzing a cell lysate in a human plasma clotting assay. The results in Table 2 show the expected upregulation by TNF of HUVEC procoagulant activity (reflected by a decreased clotting time). Chimeric antibody cA2 effectively neutralized this TNF activity in a dose-dependent manner.

TABLE 2

IN VITRO NEUTRALIZATION OF TNF-INDUCED PROCOAGULANT ACTIVITY

		<u>TNF Concentration (ng/ml)</u>		
<u>Antibody</u>	<u>ug/ml</u>	<u>250</u>	<u>25</u>	<u>0</u>
None	-	64 ± 4*	63 ± 1	133 ± 13
Control Ab	10.00	74 ± 6	N.D.	178 ± 55
cA2	10.00	114 ± 5	185 ± 61	141 ± 18
cA2	3.30	113 ± 2	147 ± 3	N.D.
cA2	1.10	106 ± 1	145 ± 8	N.D.
cA2	0.37	73 ± 17	153 ± 4	N.D.
cA2	0.12	64 ± 1	118 ± 13	N.D.

* Values represent mean plasma clotting time, in seconds (± S.D.). Clotting time was determined in normal human plasma after addition of the rhTNF (Dainippon, Osaka, Japan) with or without antibody-treated HUVEC lysate and Ca⁺⁺ at 37°C. N.D. = Not done. Control Ab is a chimeric mouse/human IgG1 anti-CD4 antibody.

In addition to stimulating procoagulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. The ability of cA2 to neutralize this activity of TNF was measured using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC were

stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37°C overnight in a 96-well plate format. surface expression of ELAM-1 was determined by sequential addition of a mouse anti-human ELAM-1 mAb and ¹²⁵I-labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 4°C.

As shown in Figure 12, TNF induced the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity was again effectively blocked in a dose-related manner by cA2.

Finally, TNF is known to stimulate mitogenic activity in cultured fibroblasts. Chimeric A2 neutralized TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent neutralizing capability of cA2 against a broad spectrum of in vitro TNF biological activities.

EXAMPLE XII

In Vivo Activity and Efficacy of cA2 Antibody

The highly restrictive species cross-reactivity of cA2 severely limits the ability to test the in vivo efficacy of this antibody in animals other than humans or chimpanzees.

Nevertheless, evidence that the potent in vitro neutralizing capability of cA2 is manifest in vivo was desirable. Earlier animal studies showed that administration of TNF to experimental

animals mimics the disease state obtained with either Gram negative bacterial infection or direct endotoxin administration (Tracey, K.J. et al., 1986. supra; Tracey, K.J. et al., 1987, supra; Lehmann, V. et al., supra).

5 An in vivo model wherein lethal doses of human TNF are administered to galactosamine-sensitized mice (Lehmann, V. et al., supra) was adapted for testing the capability of cA2 to neutralize TNF in vivo. An i.p. challenge with 5 µg (0.25 mg/kg) of rhTNF resulted in 80-90 percent mortality in untreated
10 control animals and in animals treated i.v. 15-30 minutes later with either saline or 2 mg/kg control antibody (a chimeric IgG1 derived from murine 7E3 anti-platelet mAb). In contrast, treatment with cA2 reduced mortality to 0-30 percent with 0.4 mg/kg of antibody, and to 0-10 percent with 20 mg/kgs. These
15 results, summarized in Table 4, indicate that cA2 was capable of

neutralizing the biological activity of TNF in vivo as well as in vitro.

TABLE 4

5 PREVENTION OF HUMAN TNF-INDUCED LETHALITY BY CHIMERIC A2

Outcome (Survivors/Total)

<u>Antibody</u>	<u>Experiment #1</u>	<u>Experiment #2</u>
None	1/10	N.D.
Control Ab, 2 mg/kg	2/10	1/10
10 cA2 (2 mg/kg)	9/10 (p=0.0055)	10/10 (p=0.0001)
cA2 (0.4 mg/kg)	7/10 (p=0.07)	10/10 (p=0.0001)

15 Female C3H/HeN mice were administered 5 µg rhTNF (Dainippon, Osaka, Japan) + 18 mg galactosamine i.p. and antibody was administered 15-30 minutes later i.v. Deaths were recorded 48 h post-challenge. Control MAb = chimeric mouse/human IgG1 anti-platelet MAb (7E3). N.D. = not done. p values refer to comparison with the control Ab.

20 The references cited above are, all incorporated by reference herein, whether specifically incorporated or not.

 Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

25 While this invention has been described in connection with specific embodiments thereof, it will be understood that it

is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

WHAT IS CLAIMED IS:

1. A high-affinity mouse monoclonal antibody to human tumor necrosis factor- α (TNF α) designated A2 which binds to a neutralizing epitope of human TNF α .

5 2. A mouse monoclonal antibody according to claim 1 in detectably labeled form.

3. A chimeric immunoglobulin chain comprising at least part of a human immunoglobulin constant region and at least part of a non-human immunoglobulin variable region having
10 specificity to human TNF α .

4. A chimeric immunoglobulin chain according to claim 3 which is a heavy chain.

5. A chimeric immunoglobulin chain according to claim 3 which is a light chain.

15 6. A chimeric antibody molecule comprising two light chains and two heavy chains, each of said chains comprising at least part of a human constant region and at least part of a variable region having specificity to human TNF α , said antibody binding with high affinity to a neutralizing epitope of human
20 TNF α .

7. A chimeric antibody according to claim 6 which does not bind to $\text{TNF}\beta$.

8. A chimeric antibody according to claim 6, wherein said variable region is of murine origin.

5 9. A chimeric antibody according to claim 8, wherein said variable region is derived from a high affinity murine monoclonal antibody which binds to a neutralizing epitope of human $\text{TNF}\alpha$.

10 10. A chimeric antibody according to claim 9, wherein said murine monoclonal antibody is A2 or a monoclonal antibody which competitively inhibits the binding of A2 to $\text{TNF}\alpha$.

11. A chimeric antibody according to claim 9, wherein said murine monoclonal antibody is A2.

15 12. A chimeric antibody according to claim 6 wherein said affinity, measured as an association constant (K_a), is at least 1×10^8 liter/mole.

13. A chimeric antibody according to claim 6 wherein said affinity, measured as an association constant (K_a), is at least 1×10^9 liter/mole.

14. A chimeric antibody according to claim 6 which neutralizes human TNF α with an ID50 of at least about 1 μ g/ml.

15. A chimeric antibody according to claim 6 which neutralizes human TNF α with an ID50 of at least about 100 ng/ml.

5 16. A chimeric antibody according to claim 6 which neutralizes human TNF α with an ID50 of at least about 15 ng/ml.

17. A chimeric antibody according to claim 6 in detectably labeled form.

18. A polynucleotide molecule comprising:

- 10 (a) a first nucleotide sequence encoding at least a part of the variable region of an immunoglobulin chain having specificity to human TNF α ; and
- (b) an second nucleotide sequence encoding at least a part of the constant region of a human
- 15 immunoglobulin chain,

both said sequences in operable linkage with each other, wherein said sequences are not in operable linkage with each other in nature, wherein said polynucleotide molecule encodes a protein chain capable of yielding an antibody with high affinity binding

20 to said human TNF α .

19. A molecule according to claim 18 wherein said first nucleotide sequence is a genomic DNA sequence.

20. A molecule according to claim 18 wherein said first nucleotide sequence is a cDNA sequence.

5 21. A molecule according to claim 18 wherein said second nucleotide sequence is a cDNA sequence.

22. A molecule according to claim 18 wherein said variable region is of murine origin.

10 23. A molecule according to claim 22 wherein said variable region is derived from a murine monoclonal antibody.

24. A molecule according to claim 23 wherein said murine monoclonal antibody is A2.

25. A molecule according to claim 18 wherein said immunoglobulin chain is a heavy chain.

15 26. A molecule according to claim 18 wherein said immunoglobulin chain is a light chain.

27. A molecule according to claim 18 in double stranded DNA form.

28. A molecule according to claim 18 which is an expression vehicle.

29. A molecule according to claim 28 wherein said vehicle is a plasmid.

5 30. A host transformed or transfected with the molecule of claim 28.

31. A host according to claim 30 which is a eukaryotic cell.

10 32. A host according to claim 31 which is a mammalian cell.

33. A DNA molecule comprising a DNA sequence encoding a chimeric antibody molecule according to claim 6.

34. A process for preparing a chimeric antibody according to claim 6, comprising:

- (a) culturing 'a host capable of expressing said heavy chain and said light chain under culturing conditions,
- (b) expressing said chimeric antibody; and
- (c) recovering said chimeric antibody from said culture.

35. A method for treating a subject having a disease or condition mediated by TNF α comprising administering to said subject a therapeutic amount of a monoclonal antibody according to claim 1.

36. A method for treating a subject having a disease or condition mediated by TNF α comprising administering to said subject a therapeutic amount of a chimeric antibody according to claim 6.

37. A method of removing from a fluid TNF α , a fragment thereof, or an immune complex containing said TNF α , the method comprising:

(a) delivering said fluid to a device containing a monoclonal antibody according to claim 1 in immobilized form; and

(b) allowing binding of said TNF α , fragment or immune complex to said immobilized antibody,

thereby removing said TNF α , fragment or immune complex from said fluid.

38. A method of removing from a fluid TNF α , a fragment thereof, or an immune complex containing said TNF α , the method comprising:

(a) delivering said fluid to a device containing a chimeric antibody according to claim 6 in immobilized form; and

(b) allowing binding of said TNF α , fragment or immune complex to said immobilized antibody,

thereby removing said TNF α , fragment or immune complex from said fluid.

39. A method of treating a subject suspected of having a disease or condition associated with elevated levels of TNF α in a body fluid, comprising:

- 5 (a) removing said TNF α from said body fluid using a method according to claim 38: and
(b) returning said body fluid to said subject.

40. A method of treating a subject suspected of having a disease or condition associated with elevated levels of TNF α in a body fluid, comprising:

- 10 (a) removing said TNF α from said body fluid using a method according to claim 38; and
(b) returning said body fluid to said subject.

41. An immunoassay method for detecting human TNF α in a sample, comprising:

- 15 (a) contacting said sample with a monoclonal antibody according to claim 1; and
(b) detecting the binding of the antibody to said TNF α .

42. An immunoassay method for detecting human TNF α in
a sample, comprising:

(a) contacting said sample with a chimeric
antibody according to claim 6;

5 (b) detecting the binding of the antibody to said
antigen.

ABSTRACT OF THE DISCLOSURE

High affinity neutralizing monoclonal and chimeric antibodies specific for human tumor necrosis factor- α (TNF α) are useful in diagnosis and therapy of a number of TNF α -mediated diseases and conditions, including sepsis syndrome, cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic parasitic or infectious processes, including bacterial, viral and fungal infections, acute and chronic immune and autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, alcohol-induced hepatitis, chronic inflammatory disease, vascular inflammatory diseases, graft-versus-host disease, Kawasaki's disease and malignant diseases. Polynucleotide molecules coding for the chimeric antibody, methods of producing the antibody, methods of use of the mouse monoclonal and chimeric antibody in immunoassays and immunotherapeutic approaches are provided.